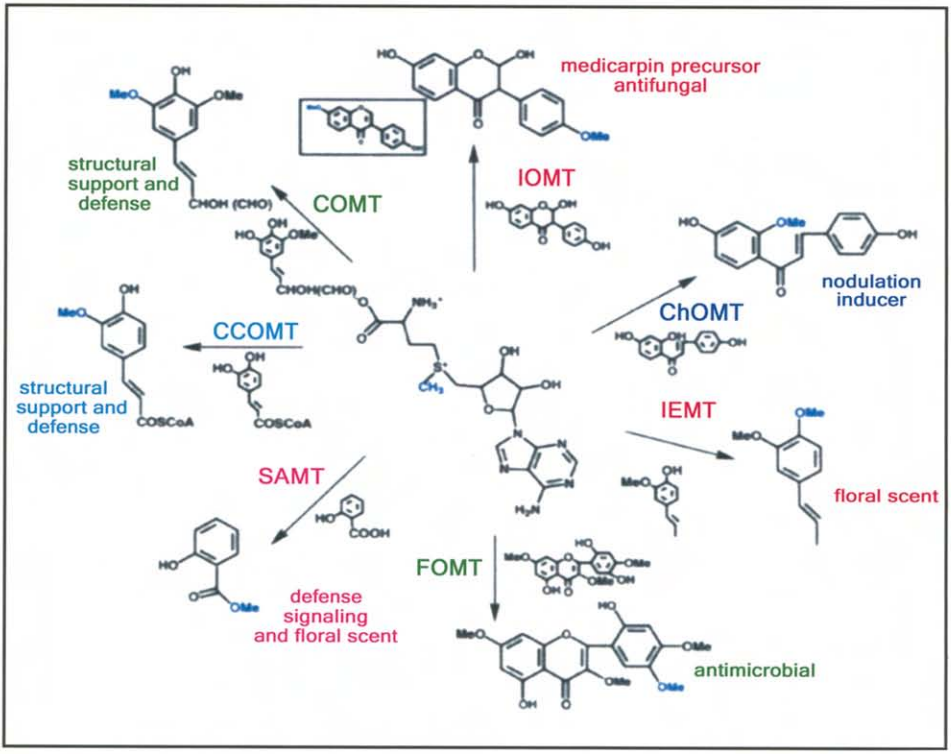
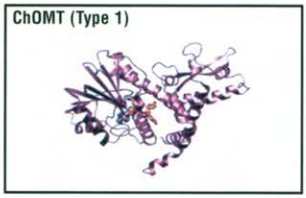
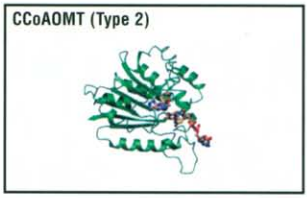
recent advances in phytochemistry – volume 37

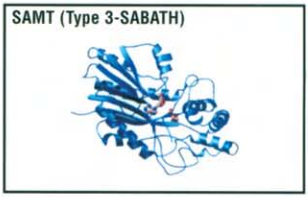
Integrative Phytochemistry: from Ethnobotany to Molecular Ecology

J.T. Romeo









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Tampa, Florida, USA

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PREFACE

The chapters in this volume are based on papers contributed at the annual meeting of the Phytochemical Society of North America held at the Hotel El Conquistador de Montejo, Mérida, Yucatán, Mexico, July 20-24, 2002. The title of the symposium was "Integrative Phytochemistry: From Ethnobotany to Molecular Ecology". The organizers were Hector E. Flores, Victor M. Loyola-Vargas, Maria de Lourdes Miranda-Ham, and Felipe A. Vazquez-Flota. Four symposia dealing with phenolics, glucosinolates, alkaloids, and terpenes were covered under the symposium umbrella topic.

Integrative biology is essentially the belief that the complexity that is biology can be understood by incorporating many perspectives from a diversity of disciplines, each of which complements the others. The papers in this volume dealing with "integrative phytochemistry" range from "ethnobotany to molecular ecology" and include chapters on the traditional phytochemical topics of synthesis, enzymology, chemical diversity, and chemical ecology of secondary metabolites. They also include less frequently covered topics in phytochemical tomes, such as cellular localization of metabolites, levels of regulation, structure/function analyses, molecular genetics, and functional genomics. Papers in the volume cover: WHAT compounds are produced; HOW they are produced; WHY they are produced; and the REGULATION that may lead to more efficient production. The chapters are grouped, as they were at the symposium, by chemical classes.

Ibrahim and Anzellotti have summarized the state of our knowledge in regard to the enzymatic reactions (hydroxylation, glycosylation, acylation, sulfation, methylation, prenylation) that contribute to the structural biodiversity of flavonoids. The continuing enhancement of the horticultural and nutritional value of plants through engineering is based on this fundamental biosynthetic knowledge. With the advent of wide scale genomic sequencing, the necessity for gene product characterization has increased enormously. Noel *et al.*, through the use of structural biology, are characterizing proteins at the atomic level. This level of functional understanding in turn is leading to a more complete appreciation of complex biosynthetic pathways by elucidating the mechanisms of individual biosynthetic reactions. The methyltransferases they study are a large and important group critical to many metabolic pathways, and the study of their substrate specificity and evolutionary divergence can potentially lead to structurally-guided metabolic engineering of pharmaceuticals, nutritional enhancers, and fragrance properties of crops. Irani *et al.*, have focused on the regulation of anthocyanin production. These bright red and blue pigments that function in a number of ecophysiological processes

are an ideal system for genetic, biochemical, and molecular dissection of complex regulatory networks, not to mention understanding the colorful world around us.

The sulfur-containing glucosinolates decompose to form more or less toxic products via action of myrosinase. Localization of glucosinoates and myrosinase has been debated for years. Using *Arabidopsis thaliana* as the model system, Adréasson and Jørgensen with data from x-ray microanalysis. TEM, immunolocalization, and *in situ* hybridization with a myrosinase probe, have shown that they are stored in adjacent cells. Wittstock *et al.*, emphasizing that the glucosinolate-myrosinase system is an important anti-herbivore defense in plants, have done a comparative study of the hydrolysis products against a diverse range of specialist and generalist herbivores. The isothiocyanate are more toxic than their corresponding nitriles among microbes, nematodes, generalist insects, and mammals, and mechanism of action studies in insects are shedding light on how specialists avoid toxicity. *A. thaliana* lines containing an epithiospecifier protein (ESP) form nitriles rather than isothiocyanates upon glucosinolate hydrolysis, and there is natural variation in hydrolysis products among ecotypes. A generalist lepidopteran herbivore feeds less on ESP producing lines. Rossiter *et al.* describe how *Arabidopsis* genomics has enabled the complex glucosinolate biosynthetic pathway to be elucidated in plants. Aphid myrosinase, the first insect purification of a myrosinase and its partial characterization, is discussed. It resembles a β-glycosidase, and its localization in non-flight skeletal muscle indicates an evolutionary defense mechanism that mimics the biochemistry of its host plant. Studies involving multitrophic interactions are underway with a probable fascinating scenario in the making.

Alkaloids, now represented by over 12,000 structures, are unique in that each group has a different biosynthetic origin. The large genomic and proteomic databases together with characterization of key biochemical functions is enabling the complete characterization of some pathways. Facchini *et al.* discuss the progress that has been made in understanding four of these groups that has come through the isolation of genes that facilitate tissue-specific and subcellular localization of biosynthesis, the determination of signal perception events, the elucidation of signal transduction pathways, and the functional analysis of gene promoters. The emphasis is on the benzylisoquinoline, terpenoid, tropane and purine alkaloids and their regulation at multiple levels. The monoterpenoid indole alkaloids are discussed in a separate chapter by De Luca. Many of these have become exploited as important treatments for neurological disorders, cancer, and for use as vasodilators. He reviews the recently characterized genes that appear to be responsible for their diversity and complexity, and focuses on the biosynthesis of vindoline in *Catharanthus roseus*. Ober, in contrast, discusses the chemical ecology of the pyrrolizidines. The point is well made that without knowledge of the major functional ecological roles of such compounds, the significance of many important discoveries from current "omics" studies may go unrecognized.

There are more than 30,000 known terpenes. The integrative efforts of those chemists are introduced in the chapter by Greenhagen *et al.* They discuss work at various levels that includes; sense and anti-sense transgenic expression and chemical ecology of aphids; the use of mutant lines to study pathogenesis and phytoalexins; cell cultures for studying enzymes of the two isoprenoid pathways; and the expression of gene sequences in heterologous hosts probing for structure-function relationships with site-directed mutagenesis and crystallographic studies. D'Auria *et al.* focus on *Arabidopsis* and a new type of methyltransferases that transfer the methyl group to an oxygen, and the SABATH family of genes that produces them. The compounds made by SABATH enzymes produce compounds with important biological functions ranging from flower scent for pollinator attraction to cellular regulators such as methyljasmonate that control a myriad of plant functions including the production of pathogenesis related proteins. Phylogenetic relationships among the SABATH genes and their functions are also being studied. Finally, the chapter by Lozoya-Gloria reminds us of our roots in ethnobotany. He discusses both the Aztec knowledge that relates to sesqi- and diterpenes from Mexican medicinal plants, as well as the use and abuse of these plants that has occurred outside or their original cultural context. The connection between the sophistication of recent work on kaurene diterpenes and their basis in pharmacological use by old Mexican cultures of "cihuaphatli" is remarkable. The anti-inflammatory mechanisms of action of several terpenoids discovered in Mexican plants are also discussed.

Those of us fortunate to attend the meeting in Mérida found it a perfect place to deliberate our specialist discipline phytochemistry in the integrative context of the symposium topic. We thank our host, Felipe Vázquez-Flota, who insured that we felt welcome and were well fed and entertained. In working on this volume, JTR is grateful for the assistance of Darrin T. King for his technical expertise and for the cooperation of the contributors.

John T. Romeo
University of South Florida

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General Editor: John T. Romeo, *University of South Florida, Tampa, Florida*

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Volume 37 **Integrative Phytochemistry: From Ethnobotany to Molecular Ecology**
Proceedings of the Forty-second Annual Meeting of the Phytochemical Society of North America, Mérida, Yucatán, Mexico, July, 2002

Cover design: Plant methyltransferases and their associated reactions. SAM in the center serves as the universal methyl donor (see Fig 2.3). Overall architectural comparisons of type 1, 2, and 3 (SABATH) plant methyltransferases rendered as backbone ribbon diagrams (see Fig 2.2).

Chapter One

THE ENZYMATIC BASIS OF FLAVONOID BIODIVERSITY

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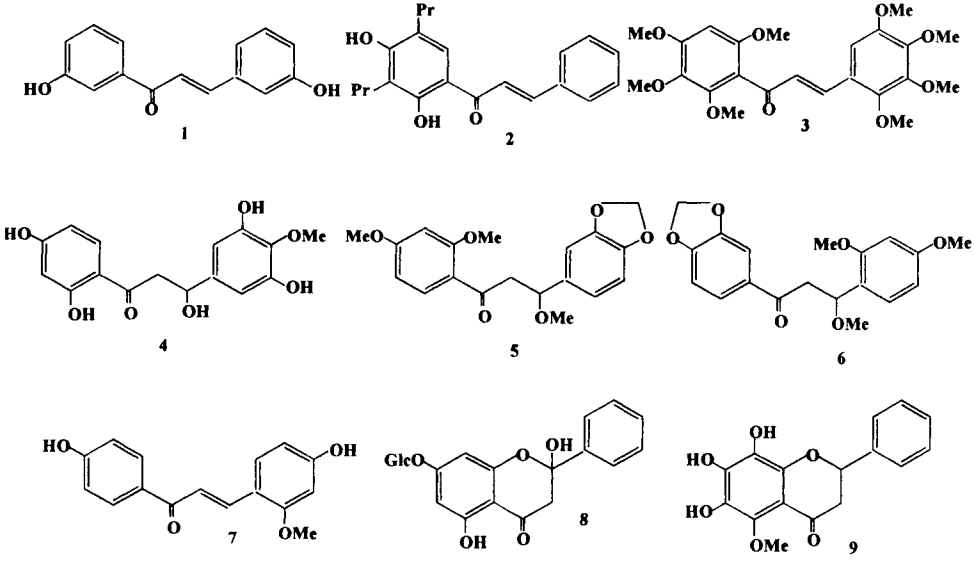
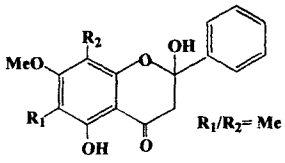
INTRODUCTION

Flavonoid compounds constitute one of the most ubiquitous groups of natural plant products. They occur in all plant organs, and are most conspicuous in flowers, leaves, and seeds by their bright colors. Flavonoids consist of two phenyl rings A and B connected by a three-carbon bridge that forms the heterocyclic ring C. Both phenyl rings are derived from the malonate/acetate and shikimate pathways, respectively. According to the oxidation level of ring C, flavonoids are classified into chalcones, flavones, isoflavones, and flavonols, as well as their dihydro derivatives, and the orange to blue colored anthocyanidins (Fig. 1.1).

Flavonoids exhibit a wide range of functions and play important roles in the biochemistry, physiology, and ecology of plants, including coloration of seeds and flower petals, pollen germination and pollen fertility, activation of *Rhizobium* nodulation genes, protection against UV radiation and pathogenic organisms, as well as roles as regulators of plant growth, enzyme inhibitors, insect antifeedants, antioxidants, and anticancer agents. They also have potential benefits to humans as nutraceuticals. These and other roles have been discussed in detail in several review articles and book chapters.[1-6]





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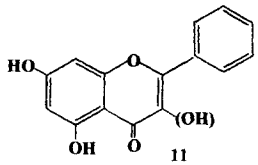

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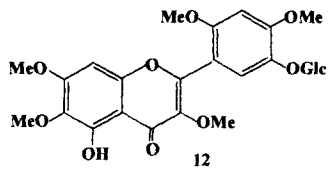

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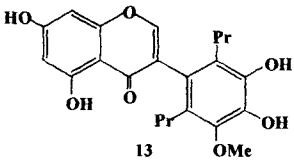

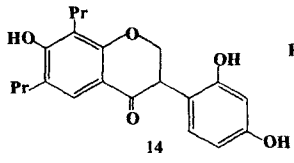

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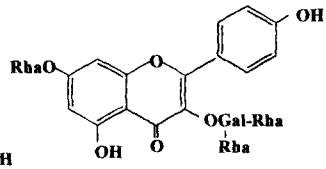

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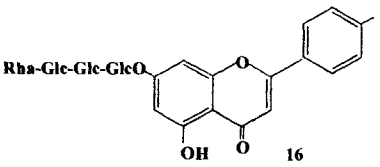

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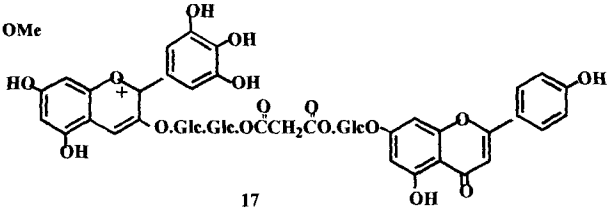

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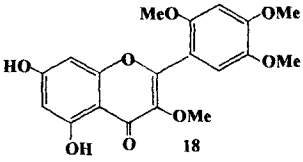

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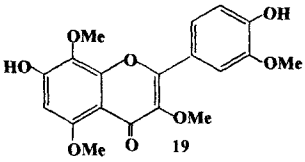

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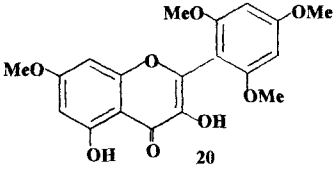

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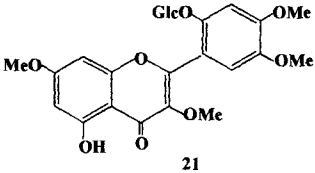

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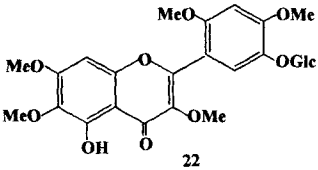

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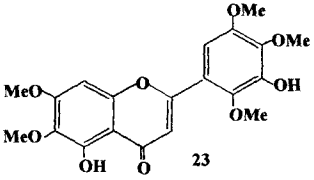

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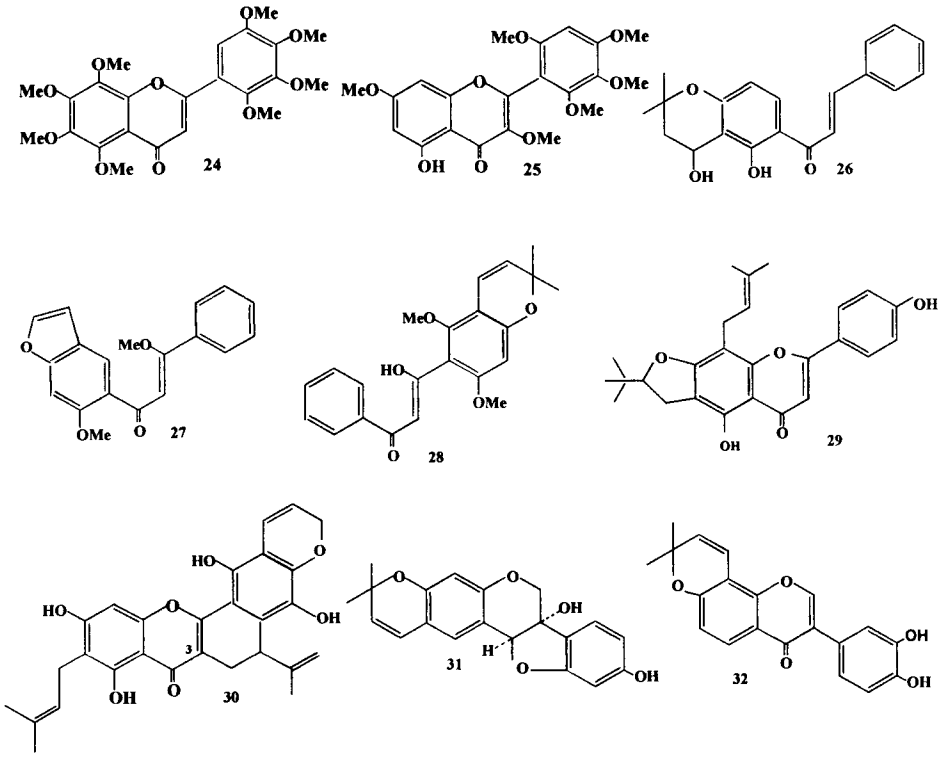


Fig. 1.1: Structures of some flavonoid compounds with unusual substitution patterns.

BIOSYNTHESIS OF FLAVONOIDS

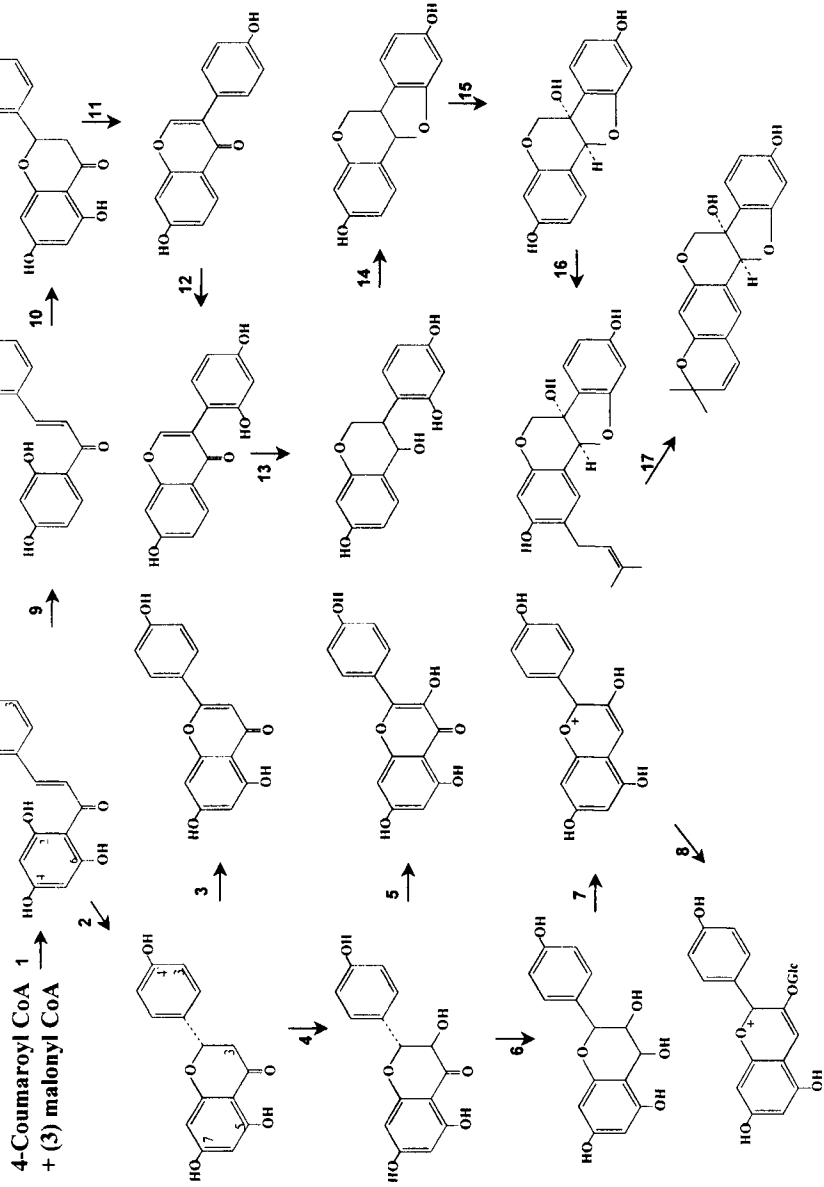
The main flavonoid skeleton derives from the stepwise condensation of three molecules of malonyl CoA with one molecule of 4-coumaroyl CoA, a reaction catalyzed by chalcone synthase (CHS) to form naringenin (2',4,4',6'-tetrahydroxy) chalcone, the common intermediate in the formation of all flavonoids with 5,7-dihydroxy (flavone numbering) A-ring substitution. In some plants, however, an NADP-dependent chalcone-ketide reductase coacts with CHS to form 6'-deoxychalcone, the precursor of 5-deoxyflavonoids. The resulting chalcones undergoe a stereospecific cyclization to the corresponding (*2S*) flavanones, the

branch point intermediates in the biosynthesis of all classes of flavonoids. This occurs by two types of chalcone isomerases (CHI): one that accepts the 6'-hydroxychalcones and the other 6'-deoxychalcones. The presence of the two types of enzymes in plants correlates with the presence of 5,7-dihyroxy (phloroglucinol-type) and 7-hydroxy (resorcinol-type) flavonoids. Flavanones may undergo either/or (a) a stereospecific hydroxylation to their corresponding dihydroflavonols by flavanone 3-hydroxylase (F3H), (b) a conversion to their corresponding flavones by the cytosolic 2-oxoglutarate-dependent flavone synthase I (FSI), or the microsomal cytochrome P450-dependent monooxygenase FSII, or (c) 2-hydroxylation, dehydration, and subsequent 2,3-aryl migration of ring B to isoflavones, a reaction catalyzed by the cytochrome P450-dependent isoflavone synthase (IFS). Both 5- hydroxyisoflavones and 5-deoxyisoflavones may be hydroxylated at the 2'-position by a microsomal isoflavone hydroxylase (IFH), and subsequently reduced by an NADPH-dependent isoflavone reductase (IFR) to their 2'-hydroxyisoflavanones. The latter may be converted to the corresponding 3,9-dihydroxypterocarpans by pterocarpan synthase (PTS). 6*a*-Hydroxylation of the latter, by a pterocarpan hydroxylase, and subsequent prenylation and cyclization of the alkyl chains result in the formation of a variety of pterocarpan phytoalexins (Scheme 1.1).

The branch pathway for anthocyanin biosynthesis starts with the enzymatic reduction of dihydroflavonols to their corresponding flavan 3,4-diols (leucoanthocyanidins) by substrate-specific dihydroflavonol 4-reductases (DFR). Flavan 3,4-diols are the immediate precursors for the synthesis of catechins and proanthocyanidins. Catechins are formed by enzymatic reduction of the flavan 3,4-diols in the presence of NADPH to leucoanthocyanidins, which are subsequently converted to anthocyanidins by the 2-oxoglutarate-dependant dioxygenase, anthocyanidin synthase. Further glycosylation, methylation, and/or acylation of the latter lead to the formation of the more stable, colored anthocyanins (Scheme 1.1). The details of the individual steps involved in flavonoid and isoflavonoid biosynthesis, including the biochemistry and molecular biology of the enzymes involved, have recently appeared in two excellent reviews.[7,8]

STRUCTURAL DIVERSITY OF FLAVONOIDS

The structural diversity of metabolites belonging to the different flavonoid classes, including their oxygenation patterns, glycosylation, sulfation, acylation, methylation, and/or prenylation are best illustrated in a recent text,[9] as well as in several earlier monographs.[10-14] These different substitution reactions, which are catalyzed by substrate-specific and position-oriented enzymes, contribute to the enormous diversity of flavonoid compounds that amount to >5000 chemical structures in Nature and hence, to the wide spectrum of functional roles they play in


4-Coumaroyl CoA 1
+ (3) malonyl CoA
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Scheme 1.1: Pathway for the biosynthesis of the major classes of flavonoids. 1, Chalcone synthase; 2, chalcone isomerase; 3, flavone synthase; 4, flavanone 3-hydroxylase; 5, flavonol synthase; 6, dihydroflavonol reductase; 7, anthocyanidin synthase; 8, anthocyanidin glucosyltransferase; 9, chalcone-ketide reductase; 10, chalcone isomerase; 11, isoflavone synthase; 12, isoflavone 2'-hydroxylase; 13, isoflavone reductase; 14, pterocarpan synthase; 15, pterocarpan 6a-hydroxylase; 16, prenyltransferase; 17, prenylcyclase.

the survival of plants. The following paragraphs present an overview of the enzymatic reactions that contribute to the structural biodiversity of this important group of natural products.

HYDROXYLATION

Except for the 5, 7, and 4'-hydroxyl groups (flavanone numbering) resulting from the condensation of 4-coumaroyl CoA and the acetate oxygens, the introduction of new hydroxyl groups on the flavonoid ring system is catalyzed by two major classes of enzymes, the monooxygenases and dioxygenases. In addition, both groups of enzymes are involved in the modification of the oxidation level of ring C, thus resulting in the different flavonoid classes.

Monooxygenases

Monooxygenases are cytochrome P450-dependent enzymes that require NADPH and oxygen for catalytic activity. They occur as integral membrane, heme-containing proteins in the endoplasmic reticulum, and constitute a superfamily of >200 plant enzymes that are involved in the biosynthesis of several groups of natural products, including flavonoids, as well as the detoxification of xenobiotics and herbicides into non-toxic products. Several reviews have dealt with their nomenclature, classification, biochemistry, molecular biology, and functional roles.[15-18]

Cytochrome P450 monooxygenases play two important roles in flavonoid biosynthesis. One involves the modification of ring C of flavanones, where they attack nonphenolic carbon atoms, thus resulting in the creation of new flavonoid classes such as flavones, flavonols, and isoflavonoids. The other involves the introduction of new hydroxyl groups on phenolic rings A and B, thus increasing the

range of hydroxylated flavonoids and their subsequent glycosylation and/or methylation. An overview of these reactions is given below.

Monooxygesases Involved in C-Ring Modifications

Flavone Synthase II (FSII)

The enzyme catalyzes the conversion of flavanones to their corresponding flavones by introducing a C2-C3 double bond. It was first demonstrated in the microsomal fraction of snapdragon flowers,[19] and more recently in soybean hypocotyls,[20] and gerbera flowers.[21] Compared with the 2-oxoglutarate-dependent FSI, which has been detected only in parsely (see a later section), FSII activity has been reported in several species and is believed to be the more common form of FS in plants.[7,22] FSII has been cloned from snapdragon[23] based on its sequence homolgy with a licorice cDNA encoding a flavanone 2-hydroxylase. It was also cloned from gerbera[24] by using a differential display technique with primers based on the conserved heme-binding region of cytochrome P450 proteins. Both the snapdragon and gerbera enzymes catalyzed the conversion of liquiritigenin to 7,4'-dihydroxyflavone and of eriodictyol to luteolin. In addition, snapdragon FSII produced small amounts of quercetin from dihydroquercetin, a reaction normally catalyzed by the dioxygenase flavonol synthase (FLS, see a later section), thus suggesting the existence of a P450-type FLS.[23] More recently, another FSII was cloned from *Perilla frutescens*[25] by using mixed heterologous probes containing the oxygen-binding pocket and heme-binding region. The microsomal recombinant protein, expressed in yeast, catalyzed the direct conversion of flavanones, such as naringenin and eriodictyol to their corresponding flavones, apigenin and luteolin, presumably via their 2-hydroxyflavanones.

Flavanone 2-Hydroxylase (F2H)

This enzyme represents the first step in the biosynthesis of flavones and isoflavones. It catalyzes the hydroxylation of 5-deoxy- and 5-hydroxyflavanones at position 2 to the hypothetical intermediates in flavone biosynthesis. F2H activity was unequivocally demonstrated by the cloning of its gene from licorice and its expression in insect cells.[26] The microsomal recombinant protein catalyzed the conversion of liquiritigenin to licodione (a reterochalcone) and both naringenin and eriodictyol to their corresponding 2-hydroxyflavanones. Upon acid treatment, they give rise to 7,4'-dihydroxyflavone, apigenin, and luteolin, respectively. F2H exhibits selectivity towards (*2S*)-naringenin for conversion to flavones. This shows that the licorice enzyme acts on three differently-hydroxylated flavanones, liquiritigenin, naringenin, and eriodictyol, and supports the hypothesis that flavone biosynthesis in

legumes proceeds via 2-hydroxylation of flavanones, as postulated for isoflavones, followed by a dehydratase-catalyzed reaction.[26]

Isoflavone Synthase (IFS)

The key enzyme in isoflavonoid biosynthesis catalyzes the oxidative 2,3-aryl migration of flavanones to isoflavones. It was first reported in soybean microsomal preparations and shown to convert both liquiritigenin and naringenin to daidzein and genistein, respectively.[27,28] It was later shown that the reaction proceeds via the 2-hydroxyflavanone intermediate with subsequent dehydration to the corresponding isoflavone.[29] cDNA clones encoding IFS were isolated from soybean[30] and licorice[31] (designated as 2-hydroxyisoflavanone synthase) microsomal preparations and expressed in yeast and insect cells, respectively. Both recombinant proteins convert 4',7-dihydroxyflavanone to daidzein, most likely via the 2,4',7-tri-hydroxy-isoflavanone, which spontaneously dehydrates to daidzein. The enzyme also converts naringenin to genistein, albeit to a smaller extent, which corresponds with the accumulation of daidzein in the seeds of fungus-infected soybean.[28] More recently, IFS has been cloned from several legumes, as well as the non-leguminous sugar beet.[32] Expression of IFS in *Arabidopsis*, which does not produce isoflavones, led to the accumulation of genistein, thus allowing the manipulation of the phenylpropanoid pathway for agronomic purposes.[32]

Monooxygenases Catalyzing New Hydroxylations

These are typical microsomal cytochrome P450-dependent, mixed-function monooxygenases that require NADPH and oxygen for catalytic activity, and are inhibited by the typical P450 inhibitors.[16-18] They catalyze the introduction of new hydroxyl groups on flavonoid rings A and B as well as the pterocarpanoid ring D, thereby increasing the range of flavonoid oxygenation and hence structural diversity.

Flavonoid 3'-Hydroxylase (F3'H)

This enzyme was first reported in the microsomal fractions of *Haplopappus gracilis* cell cultures,[33] studied in more detail in parsley,[34] and later shown to occur in the flowers of several species.[7] F3'H catalyzes the hydroxylation of naringenin and dihydrokaempferol (DHK), as well as of apigenin or kaempferol to their respective 3'-hydroxy derivatives, eriodictyol, dihydroquercetin (DHQ), luteolin, and quercetin, but does not accept the flavan 3,4-diols or anthocyanidins as substrates,[34] indicating that B-ring hydroxylation of the latter is determined at the dihydroflavonol level. It was more than two decades later that the first cDNA clone encoding F3'H was isolated and characterized from the flowers of *Petunia hybrida,*[35] *Arabidopsis thaliana,*[36] and *Perilla frutescens.*[37] Their recombinant proteins were shown to

catalyze the 3'-hydroxylation of naringenin, DHK, and, to a lesser extent, of apigenin and kaempferol. The fact that F3'H does not accept *p*-coumaric acid as substrate supports the long held view that flavonoid B-ring oxygenation pattern is established at the C_{15} rather than the substituted hydroxycinnamate level.

Flavonoid 3'/5'-Hydroxylase (F3'/5'H)

This enzyme catalyzes the hydroxylation of naringenin and DHK at both positions 3' and 5', as well as eriodictyol and DHQ at position 5' to give rise to pentahydroxyflavanone and dihydromyricetin, respectively.[7,38] The latter compounds are intermediates in anthocyanidin biosynthesis and play an important role in determining flower color, especially after the methylation of B-ring hydroxyls. A cDNA encoding F3'/5'H was isolated from the flower petals of *Petunia hydbrida*[39] and from UV-irradiated eggplant seedlings.[40] The flux through the anthocyanin pathway appears to be modulated by the presence/absence of F3'H and F3'/5'H and their relative activities, and, therefore, their genes are considered of great biotechnological importance in modulating flower color in plants.[41]

It has recently been reported that cytochrome b_5 is required for full activity of F3'/5'H. The gene encoding this protein, *difF*, was cloned from petunia[42] and shown to be exclusively expressed in flowers generating purple or blue petals. Inactivation of the gene reduced both F3'/5'H activity and 5'-substituted pigments, thus resulting in altered flower color.[42]

Isoflavone 2'- and 3'-Hydroxylases (IF 2'/3'H)

Microsomal preparations from yeast-elicited cell cultures of chickpea catalyzed the hydroxylation of formononetin and biochanin A to their 2'- and 3'-hydroxy derivatives.[43,44] Neither daidzein nor genistein were accepted as substrates. Both hydroxylation reactions seem to be catalyzed by two distinct enzymes, since they exhibited different physicochemical properties and induction kinetics in cell cultures and roots of chickpeas.[45] Both 2'- and 3'-hydroxylations of formononetin are prerequisite reactions in the pathway for biosynthesis of the phytoalexins, medicarpin and maackiain in alfalfa and chickpea, respectively[8].

Chalcone 3-Hydroxylase (CH3H)

Microsomal preparations of *Dahlia variabilis* flowers have been reported to catalyze the 3-hydroxylation of the 6'-deoxychalcone, isoliquiritigenin, to butein.[46] This enzyme activity was shown to be a typical cytochrome P450 monooxygenase that appears to be different from the F3'H, although its occurrence in other species remains to be investigated.

Flavonoid A-ring Hydroxylases

Microsomal preparations from flowers of *Tagetes patula* and *Chrysanthemum segetum* were reported to catalyze the hydroxylation of quercetin to quercetagetin (6-hydroxyquercetin) or gossypetin (8-hydroxyquercetin) by typical cytochrome P450-dependent monooxygenases.[47] Both enzyme activities correlated with the natural occurrence of quercetagetin- and gossypetin 7-*O*-glucosides in both sources, respectively. However, it remains to be determined whether both hydroxylations are catalyzed by one or two, position-specific enzymes.

More recently, a cDNA clone encoding a cytochrome P450-dependent hydroxylase was isolated from elicitor-induced soybean cells and expressed in yeast.[48] The microsomal recombinant protein converted both 5-deoxy- and 5-hydroxyflavanones to their 6-hydroxy derivatives, more efficiently than either flavones, dihydroflavonols, or flavonols (chalcones or dihydrochalcones were not tested). The enzyme did not accept isoflavones as substrates in spite of the natural occurrence of 6,7-dioxygenated isoflavones in soybean. However, the fact that 6,7,4'-trihydroxyisoflavanone was converted to its isoflavone derivative, by a recombinant 2-hydroxyisoflavanone synthase, suggests that 6-hydroxylation occurs before aryl migration in isoflavone biosynthesis.[48] It appears, therefore, that the soybean enzyme is involved in ring-A hydroxylation of isoflavones, which correlates with the occurrence in the plant of glycitein (6-methoxy-7,4'-dihydroxyisoflavone) and afrormosin (7-hydroxy-6,4'-dimethoxyisoflavone).

Pterocarpan 6a-Hydroxylase (PT6aH)

A feature of some pterocarpan phytoalexins (*e.g.*, pisatin and glyceollin of pea and soybean, respectively) is their hydroxylation at position 6*a*, a reaction catalyzed by a microsomal cytochrome P450 monooxygenase.[49,50] A cDNA encoding this enzyme was recently characterized from elicited soybean cell cultures.[51] The microsomal protein, expressed in yeast cells, catalyzed the stereoselective hydroxylation of (6*aR*, 11*aR*)-3,9-dihydroxypterocarpan to its 6a-hydroxy derivative. It was also demonstrated that the enzyme expression is regulated at the transcriptional level.[51]

Dioxygenases

Dioxygenases are nonheme, iron-containing cytosolic enzymes that require 2-oxoglutarate (2-OG), Fe^{2+} and ascorbate for catalytic activity. They are involved in the biosynthesis of various metabolites, including the growth regulators, abscisic acid, ethylene, and some gibberellins, as well as numerous secondary metabolites. They catalyze a variety of enzymatic reactions in flavonoid biosynthesis such as desaturation (flavone synthase I, flavonol synthase), oxidation (anthocyanindin

synthase), and hydroxylation (flavanone 3-hydroxylase, partially methylated flavonol 6-hydroxylase). The biochemistry, genetics, and molecular biology of the most common, 2-OG-dependent dioxygenases have been thoroughly reviewed,[52-54] and only a brief account of those enzymes involved in flavonoid synthesis is given below.

Dioxygenases Involved in C-Ring Modifications

Flavone Synthase I (FSI)

In contrast with the widely distributed cytochrome P450-dependent FSII, FSI has been reported only once, in the young leaves of parsley (*Petroselinum crispum*), to convert flavanones to flavones,[55] although FSI may be prevalent in the Apiaceae family. The fact that 2-hydroxynaringenin was neither accepted as a substrate, by a highly purified enzyme preparation, nor did it inhibit flavone formation ruled out its being an intermediate in flavone synthesis.[56] It is now assumed that the reaction mechanism of FSI involves the sequential abstraction of hydrogen atoms from C-2 and C-3 with the formation of a double bond. A cDNA encoding FSI was recently isolated and identified from parsley,[57] and its recombinant protein, expressed in a yeast system, was shown to convert naringenin to apigenin, and exhibited no flavonol synthase activity.

Flavonol Synthase (FLS)

This enzyme catalyzes the stereospecific conversion of the (*2R, 3R*)-dihydroflavonols, DHK and DHQ, to the corresponding flavonols, by introducing a double bond between C-2 and C-3. The enzyme was first reported in parsley cell cultures,[58] and later shown to occur in several plant species.[7] A cDNA clone encoding FLS was isolated from *Petunia hybrida,*[59] and its antisense expression strongly reduced flavonol synthesis in flower petals, thus allowing a higher flux of dihydroflavonols to be channeled towards anthocyanin synthesis.[59]

Anthocyanidin Synthase (ANS)

This enzyme catalyzes the conversion of flavan 3,4-diols (leucoanthocyanidins) to their corresponding anthocyanidins.[60] A cDNA encoding ANS was recently isolated from *Perilla frutescens*,[61] and its recombinant protein catalyzed the oxidation of both leucocyanidin and leucopelargonidin to their corresponding anthocyanidins, most likely via their 2-flaven-3,4-diols upon subsequent acidification. The enzyme exhibited a 3-fold higher affinity for leucocyanidin over leucopelargonidin.[61] Leucodelphinidin was not tested as a substrate.

Dioxygenases Catalyzing New Hydroxylations

Flavanone 3-Hydroxylase (F3H)

The enzyme catalyzes the stereospecific 3 β-hydroxylation of (2S)-flavanones to their corresponding (*2R, 3R*)-dihydroflavonols.[62] F3H exhibits different substrate specificities in different plant species, *e.g.*, 5,7,3',4',5'-pentahydroxyflavanone was converted efficiently to dihydromyricetin by *Verbena*, but not by *Petunia* enzyme extracts.[63] F3H is considered of pivotal importance in the regulation of flavonoid biosynthesis. Its expression is coordinated with different subsets of flavonoid enzymes in different plant species,[64] and is correlated with the presence of flavonoids at the different sites of its expression.[65] Genomic and/or cDNA clones were isolated and characterized from several plant species[7], including *Petunia*,[66] and shown to exhibit high amino acid sequence homologies and conserved regions among widely divergent plant species.[67]

Partially Methylated Flavonol 6-Hydroxylase (PMF6H)

In contrast with the microsomal, cytochrome P450-dependent flavonol 6-hydroxylase of *Dahlia*[46] and the flavonoid 6-hydroxylase of soybean,[48] 6-hydroxylation of partially methylated flavonols (PMFs) is catalyzed by a cytosolic, 2-OG-dependent dioxygenase.[68] The enzyme was recently purified to near homogeneity from *Chrysosplenium americanum* shoot tips and shown to exhibit strict specificity for position 6 of PMFs possessing a 7-methoxyl group, with the highest preference for 3,7,4'-trimethylquercetin as a substrate. The fact that the hydroxylase reaction could be coupled with a Mg^{2+}-dependent 6-*O*-methyltransferase indicated its involvement in the biosynthesis of polymethylated flavonol glucosides in this plant.[69] A cDNA encoding PMF6H was isolated and characterized; its amino acid sequence exhibits the highest homology (47%) to the F3H from *Juglans nigra*, and the dioxygenase motifs characteristic of this class of enzymes are present in the carboxy terminal portion of the protein (Anzellotti and Ibrahim, unpublished data).

Flavonoid Oxygenation Patterns

Whereas some flavonoids may lack one or more hydroxyl groups from the usual 5,7,4'-oxygenation pattern of naringenin, others may acquire new hydroxyls through the action of some of the hydroxylases mentioned above. These commonly occur at positions 6, 8, 2', 3', and/or 5' and may be further substituted. On the other hand, 2-hydroxylated flavonoids are of rare occurrence. The following examples chosen from the different flavonoid classes demonstrate both phenomena, although

newly introduced hydroxyl groups may undergo further *O*-methylation and/or *O*-glycosylation.

Chalcones and Dihydrochalcones

2',4'-Dihydroxychalcone, its 2'-methyl ether and 2',4'-dihydroxy-3'-methoxychalcones in *Acacia neovernicosa,*[70] 2'-methoxy-4'-prenyloxychalcone, 2',4'-dihydroxy-3'-*C*-prenylchalcone and its 4'-methoxy derivative in *Helichrysum regulosum,*[71] 3'-3-dihydroxychalcone (**1**) in *Primula macrophylla,*[72] and 2',4'-dihydroxy-3',5'-di-*C*-prenylchalcone (**2**) in *Tephrosia spinosa,*[73] represent examples of the unusual oxygenation patterns of rings A and/or B. Examples of extra hydroxyls include the 2',4',2,4,5-pentahydroxy-3'-*C*-prenylchalcone in *Crotalaria ramosissima,*[74] 2',4'-dihydroxy-3',6',2-trimethoxychalcone in *Scutellaria luzonica,*[75] and 2',3',4',6',3,4,5,6, octamethoxychalcone (**3**) in *S. Indica.*[76] Whereas dihydrochalcones with a lack, or a high level, of oxygenation are less represented, the following examples with an extra β-oxygenation are noteworthy: 2',4',3,5, β-pentahydroxy-4-methoxydihydrochalcone (**4**) in *Gliricidia sepium,*[77] and 4',6', β-trimethoxy-3,4-methylene dioxydihydrochalcone (**5**) and β,2,4-trimethoxy-4',5'-methylenedioxy dihydrochalcone (**6**) in *Millettia hemsleyana.*[78] A small group of chalcones is known whose A- and B-ring oxygenation patterns appear to be reversed (retrochalcones). This was first identified in *Glycyrrhiza echinata* cell cultures as the 4',4-dihydroxy-2-methoxychalcone (**7**), echinatin,[79] and it was shown that ring A with its oxygen at the *para*-position was formed from phenylpropanoid precursors, as would be expected, and ring B bearing oxygens on alternate carbons derived from acetate.[79]

Flavanones

7-Hydroxyflavanone represents the simplest compound of this class and is found in many legumes.[9] 2-Hydroxyflavanones, which are expected to be quite labile and converted to their corresponding flavones, have also been identified. These include the 2,5,7-trihydroxyflavanone 7-*O*-glucoside (**8**) in *Malus spp.,*[80] 2,5-dihydroxy-7-methoxyflavanone in *Populus nigra,*[81] 6,7,8-trihydroxy-5-methoxyflavanone (**9**) in *Isodon oresbius,*[82] and 2,5-dihydroxy-7-methoxyflavanone and its 6-C- and 8-C-methyl isomers (**10**) in *Friesodielsia enghiana.*[83] All of these exhibit a lack of B-ring hydroxylation.

Flavones and Flavonols

Apart from the common 4'-, 3',4'- and 3',4',5'-oxygenated flavones and their flavonol analogs, the less common 5,7-dihydroxyflavone (**11**) and the more common

3,5,7-trihydroxyflavone (**11**) represent those that lack B-ring oxygenation. A-ring hydroxylation of flavones and flavonols at positions 6 and or 8 is not uncommon, and may be associated with a 2',4'-di- or 2',4',5'-trioxygenation (**12**) of 6-hydroxyquercetin (quercetagetin).[84]

Isoflavones and Isoflavanones

Isoflavones exhibit the same oxygenation pattern as flavones, and range from the simple (5-deoxy) to more complex structures where nearly all positions are substituted with hydroxyl, methoxyl, and/or prenyl groups (**13**).[85,86] Although isoflavanones are smaller in number than isoflavones, their structural complexity is in no way reduced, as appears in (**14**).

Anthocyanidins

Deoxyanthocyanidins are less common in flowers. However, compounds with hydroxyl groups at position 4' (pelargonidin), 3',4' (cyanidin), and 3',4',5' (delphinidin), as well as their methyl ether derivatives, are the most ubiquitous flower pigments. 6-Oxygenation occurs in a few cases. Enzyme study of the latter hydroxylation step is lacking, as is any report on the natural occurrence in plants of 6-hydroxydihydroflavonols or their flavan derivatives, the presumed precursors of 6-hydroxyanthocyanidins. Anthocyanidins are notorious for the complexity of their glycosylation and/or acylation patterns (see following section).

GLYCOSYLATION

Because of their toxicity and the reactivity of their hydroxyl groups, most flavonoids are bound to one or more sugars, commonly glucose, galactose, arabinose, rhamnose, xylose, glucuronic acid, and/or galacturonic acid. Flavonoid-sugar binding is catalyzed by a multigene family of glycosyltransferases that utilize UDP- or TDP-activated sugars to form the corresponding *O*- or *C*-glycosides. Glycosylation inactivates the reactive hydroxyl groups of flavonoids and increases their solubility and access to active transport systems. Except for a few glycosyltransferases that are substrate and position specific,[87-89] the broad substrate specificity reported for this family of enzymes[7,90] may be due, in part, to difficulty in achieving their purification to near homogeneity. However, the recent cloning of their genes and purification of their recombinant proteins allowed a thorough characterization of their substrate preferences, especially the long-disputed UDP-glucose: anthocyanidin-[91] and UDP-glucose:flavonol[92] 3-*O*-glucosyltransferases of *Vitis vinifera* and *Petunia hybrida*, respectively. The latter was used as a heterologous probe to isolate and characterize a cDNA clone encoding a flavonoid 7-*O*-glucosyltransferase from *Scutellaria baicalensis* hairy roots.[93] The recombinant

protein catalyzed the 7-*O*-glucosylation not only of baicalein (5,6,7-trihydroxy flavone), but also of apigenin and kaempferol, which share similar, though not identical, oxygenation patterns.

Molecular phylogenetic analysis of the glycosyltransferase multigene family of 117 sequences in *Arabidopsis thaliana,*[94] one of the largest in this plant, showed that 88 unique complete genes fall into 12 major groups that are associated with the glycosylation of plant hormones, and secondary metabolites. Three of these are known to be involved in 3-*O*-glucosylation of anthocyanidins, one in transferring rhamnose to the 3-glucose residue, and another in transferring glucose to the 5-position.[94]

The structural variations among flavonoid glycosides owe their complexity to a combination of factors that involve (a) the various flavonoid classes as sugar acceptors, (b) the number of positions available for glycosylation, (c) the type of sugar involved and its form: α/β or pyranose/furanose, (d) the level of glycosylation, *i.e.*, mono-, di- or triglycosides, and (e) the position of *O*-linkages in di- or triglycosides. Most favored positions for flavonoid glycosylation are 2', 4', or 6' in chalcones, 7 in flavanones and flavones, 3 and 7 in dihydroflavonols and flavonols, and 3 and 5 in anthocyanidins. As a result, there is a myriad of naturally occurring flavonoid glycosides. These can be found in the different chapters devoted to each class in "The Flavonoids – Advances in Research" series.[11-13] The following examples represent some unusual glycosides belonging to some flavonoid classes. Of rare occurrence are the 2'- and 5'-glucosides of partially methylated flavonols in *Chrysosplenium americanum.*[84] A kaempferol tetraglycoside[95] represents the structural complexity of different sugar residues and different points of attachement (**15**), as compared to a flavone tetraglycoside,[96] where all of the sugars are attached to the same flavonoid position (**16**). Among the anthocyanins with a complex glycosylation pattern is a delphinidin 3-*O*-gentiobioside linked to apigenin 7-*O*-glucoside through a bifunctional malonic acid residue[97] (**17**) and a polyacylated delphinidin glycoside with a variety of aliphatic and aromatic acyl linkages.[98]

Flavonoid C-Glycosides

It was only in the 1960s that a new class of glycosides came to be recognized in which the sugar residues, commonly glucose, arabinose, rhamnose, or xylose, are attached directly to the A-ring carbon atoms 6 and/or 8. *C*-Glycosyl derivatives of flavones are the most common, although those of flavanones, isoflavones, dihydroflavonols, and flavonols are also known. Their chemistry, occurrence, and biosynthesis have been reviewed.[99,100] The most common isomeric *C*-glycoflavones are vitexin (8-*C*-glucosylapigenin), isovitexin (6-*C*-glucosylapigenin), orientin (8-*C*-glucosylluteolin), iso-orientin (6-*C*-glucsyloluteolin), lucenin-1 (6-*C*-xylosyl-8-*C*-glucosylluteolin), and vicenin-3- (6-*C*-glucosyl-8-*C*-xylosylluteolin). One unusual

member of this class, 3,6,8-tri-*C*-xylosylapigenin, has been reported from the fern, *Asplenium viviparum*.[101]

It has been shown, though only once, that 2-hydroxynaringenin and UDP-glucose are the respective substrate and sugar donor for the 6-/8-*C*-glucosyltransferase of *Fagopyrum esculentum*, and that subsequent dehydration of the enzyme reaction products leads to the corresponding flavone *C*-glucosides, isovitexin and vitexin.[102,103] The natural occurrence of a variety of flavonoid *C*-glycosides should entice further studies on the substrate- and regio-specificity of this class of glycosyltransferases.

ACYLATION

Most flavonoids, especially flavonol glycosides and anthocyanins, are acylated with aliphatic (acetic, malonic, succinic) or aromatic (hydroxybenzoic, hydroxycinnamic) acids on one or more hydroxyl groups of the sugar moiety or directly on the flavonoid hydroxyl groups. This reaction, which is catalyzed by a family of multifunctional acyltransferases, has been reported in several plant species[7,104,105] with a defined specificity for the flavonoid glycosides, the sugar residue, and the coenzyme A esters of aliphatic or aromatic acids involved. Aromatic, but not aliphatic, acylation of anthocyanins causes intra- and/or intermolecular copigmentation, thus rendering them more stable and bluer in color; an effect that is extended with the increasing number of acyl groups.[106]

cDNAs encoding anthocyanin 3-glucoside-[107] and 5-glucoside[108] aromatic acyltransferases and anthocyanin 5-*O*-glucoside malonyltransferase[109] have been isolated and characterized in *Perilla frutescens*, *Salvia splendens,* and *Gentiana triflora*, respectively. Analysis of the open reading frames of the sequences revealed motifs that are highly conserved among homologous members of the multifunctional acyltransferase superfamily.[110] These and other similar genes can be useful in studying the structure-function relationships of acyltransferases, as well as their potential use as molecular tools to modify anthocyanin structures and hence, expand the range of flower colors.

SULFATION

Flavonoid sulfation is the most recently recognized conjugation reaction, being catalyzed by a small family of cytosolic sulfotransferases (STs) that utilize 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as the sulfate group donor with flavones, flavonols, or their methyl ethers as acceptor molecules. Although sulfated flavonoids were considered of common occurrence by 1975,[111] their enzymology lagged behind for several years until methods were developed for the synthesis of specifically sulfated flavonoids[112] that could be used as substrates and reference

products, as well as for the development of an accurate, rapid enzyme assay[113] that was based on the formation of an ion pair between tetrabutyl ammonium dihydrogen phosphate and the sulfated reaction product. This allowed the demonstration, for the first time, of ST activity in cell-free extracts of *Flaveria bidentis*[114] and the existence of a family of enzymes involved in polysulfate synthesis. Four position-specific flavonol STs were isolated and characterized from *F. chloraefolia*[115] and *F. bidentis,*[116] and the sequential order of enzymatic sulfation of flavonol tetrasulfate was envisaged to start with the sulfation of quercetin at position 3 by 3-ST.[117] This was followed by sulfation at positions 3' (in *F. bidentis*) or 4' (in *F. chloraefolia*) of quercetin 3-sulfate by a 3'/4'-ST, and then at position 7 of the 3,3'- or 3,4'-disulfates, and finally a 3'/4'-ST that gives rise to quercetin 3,7,3',4'-tetrasulfate,[118] the major flavonoid in *F. bidentis*. There are no studies on other STs involved in the sulfation of other positions, *e.g.*, 6 and 8 of flavonols, or of the various sugars involved in the biosynthsis of sulfated glycosides.

cDNA clones encoding the flavonol 3- and 4'-STs have been isolated,[119] and their expressed fusion proteins have been shown to possess the same substrate and position specificities as those isolated from *Flaveria* species. Amino acid sequence alignment of *Flaveria* STs with other cytosolic STs allowed the recognition of conserved regions that participate in shared functions, such as cosubstrate (PAPS) binding and catalysis.[120] In addition, the construction of chimeric proteins between the flavonol 3- and 4'-STs allowed the designation of domains that are responsible for the substrate and position specificities.[121] More recently, a flavonoid ST cDNA was isolated and characterized from *A. thaliana*, and its recombinant protein exhibited strict specificity for position 7 of flavones, flavonols, and their monosulfates, with the highest affinity for kaempferol 3-sulfate> isorhamnetin> kaempferol> quercetin> apigenin 4'-sulfate> apigenin (S. Gidda & L. Varin, pers. commun.). Although *Arabidopsis* is not known to accumulate flavonoid sulfates, its 7-ST is different from that of *F. bidentis,* which accepts only a flavonol disulfate[116] on the pathway for tetrasulfate biosynthesis.

It has been proposed[110] that sulfation of flavonoids functions in the detoxification of their reactive hydroxyl groups and increases their solubility for storage in hydrophilic cellular compartments. In addition, their accumulation in plants growing in saline or marshy habitats is considered an ecological adaptation in the sequestering of sulfate ions. Recently, however, it has been shown that metabolite sulfation plays an important role in intermolecular recognition and signaling in a variety of biological processes, such as the seismonastic and gravitropic movements of plants, early nodule initiation in *Rhizobium meliloti*-alfalfa interaction by Nod RM1, and the regulation of auxin levels in plant growth.[118]

Recent reviews[122-124] have reported the natural occurrence of more than 150 sulfated flavonoids in some 300 species belonging to several dicot and monocot families. These are mostly mono- to tetrasulfate esters of common hydroxyflavones and hydroxyflavonols and their methyl ethers and less commonly of their

glycosylated derivatives, where the sulfate group may be linked to the 3- or 6-position of the sugar moiety. Because of lability of the sulfate ester bond, especially in acid media, many sulfated flavonoids may have previously escaped detection, and, sulfate esters of flavonoid classes other than flavones and flavonols have yet to be described.

The most common flavonoid sulfates are well represented in plants with sulfation at positions 7>>3'>4'>6>8 in flavones and positions 3>>7>4'>3' in flavonols. Furthermore, *O*-methylation and/or *O*-glycosylation of the aglycones add to their complexity and structural diversity among the sulfated flavonoids.[122] Apart from the common flavone monosulfates, apigenin also occurs as the 7-sulfatoglucoside, -galactoside, and –glucuronide. Luteolin is found as the 7-sulfate 3'-glucoside/rutinoside and 7-sulfatorutinoside, as well as the 6- or 8-hydroxy/methoxy luteolin mono- or disulfates. Tricetin, on the other hand, occurs as the 7-sulfatoglucoside or glucuronide, as well as the disulfatoglucuronide. In addition, the monosulfate and sulfatoglucoside/rutinoside derivatives of the *C*-glycoflavones, vitexin, isovitexin, orientin, and isoorientin, have been reported.[122] Of the flavonol aglycones, kaempferol and its 6-hydroxy/methoxy derivatives occur as the mono-, di-, or trisulfates, as well as the 3-sulfatoglucoside, -rutinoside, and –gentiobioside. Quercetin is invariably sulfated at all positions except the 5-hydroxyl group, whereas its 6- or 8-hydroxy derivatives and their mono-/dimethyl ether derivatives occur as the mono-, di-, or trisulfates, as well as sulfatoglucoside, -rhamnoside, or –rutinoside. These are just a few examples depicting the structural variations among the two major classes of sulfated flavonoids; a more complete listing can be found in several reviews.[122-124] There have been no studies aimed at determining the sequence of the enzymatic reactions involving the glycosylation, methylation, and sulfation of these compounds – a challenging task !

METHYLATION

Flavonoid *O*-methylation is catalyzed by a family of *S*-adenosyl-L-methionine (AdoMet)-dependent methyltransferases (OMTs). They catalyze the transfer of the methyl group of AdoMet to an acceptor molecule with the concomitant formation of the corresponding methyl ether derivative and *S*-adenosyl-L-homocysteine (AdoHcy) as products. Enzymatic methylation of flavonoids inactivates the reactivity of the hydroxyl groups and alters the solubility and, hence, the intracellular compartmentation. Flavonoid OMTs are both substrate- and position-specific enzymes, as was shown with the stepwise methylation catalyzed by five distinct enzymes (3 → 3,7 → 3,7,4' → 3,7,4',5'- or 3 → 3,7 → 3,7,4' → 3,6,7,4' → 3,6,7,2',4'-OMTs) involved in the biosynthesis of polymethylated flavonol glucosides in *Chrysosplenium americanum*.[125,126] Most of the so-called OMTs with broad substrate specificity[7] were reported during the 1970s and early 1980s when

few attempts were made to purify these enzymes to a high degree. Molecular cloning and expression of a number of flavonoid OMTs indicates that, in fact, these enzymes exhibit both substrate and position specificities.[127]

Enzymatic *O*-methylation occurs with all classes of flavonoids,[7] including catechins,[128] and on all positions including the 5-hydroxyl group of isoflavones,[129] which is usually chelated with the C-ring carbonyl group, and the uncommon positions 6, 8, and 2' of flavones and flavonols.[125,126,130] Stepwise *O*-methylation of flavonols by distinct OMTs has been described in spinach leaves,[131] apple cell cultures,[132] and *Chrysosplenium* shoots,[69] where *O*-methylation at position 3 was the first step of the sequence, followed by positions 7 and 3'/4', then 6. Flavonoid OMTs usually utilize the aglycones as substrates, although the gluco/glycosides may act as methyl acceptors, as with the vitexin 2" *O*-rhamnoside 7-OMT in *Avena sativa*,[133] the partially methylated flavonol 2'- and -5'-*O*-glucosides for further methylation at positions 5' and 2', respectively, in *C. americanum*,[125] and the cyanidin- and delphinidin 3-*O*-(*p*-coumaroyl)-rutinoside-5-*O*-glucoside for methylation at positions 3' and 3'/5', respectively, in *Petunia hybrida*.[134]

Except for anthocyanins, OMT cDNA clones have been isolated and characterized for almost all classes of flavonoids, including those specific for positions 3 of stilbenes,[135] 2' of chalcones[136] and retrochalcones,[137] 7 of flavanones,[138] flavones,[139] and isoflavones,[140] 3' of flavonols,[141] 3'/5' of partially methylated flavonols,[142] and 3 of pterocarpans.[143] Amino acid sequence alignment of these clones shows five consensus motifs located within the third part of the sequences distal to the carboxy terminal.[127] Most often, the degree of sequence homology among the different OMTs is related to the extent of similarity/identity in their consensus motifs. However, since the majority of the OMT clones have been isolated from plants that were induced with abiotic or biotic elicitors, they may not be representative of these classes of flavonoids. Therefore, the study of more flavonoid OMT genes will allow us to distinguish the amino acid sequences responsible for the substrate specificity of their gene products, as well as the demonstration of their phylogenetic relationships.[127]

Apart from the common mono- to trimethylated compounds, most hexa- to octahydroxy flavones appear to be highly *O*-methylated, possibly as a means of detoxification of the large number of their reactive hydroxyl groups. Among these are the methyl ethers of flavanones, flavones, and flavonols that carry methoxyl groups at positions 3,2',4',5' (**18**) and 3,5,8,5' (**19**) in *Gymnosperma glutinosa*,[144] 7,2',4',6' (**20**) in *Artocarpus heterphyllus*,[145] 3,7, 4',5' (**21**) and 3,6,7,2',4' (**22**) in *Chrysosplenium americanum*,[84] where 2'- and 5'-glucosylations occur prior to 5'- and 2'-methylations, respectively.[126] Other highly *O*-methylated derivatives include methoxyl substitutions at positions 6,7,2',4',5' (**23**) in *Brickellia glutinosa*,[146] 3,7,2',4',6' (**24**) and 5,6,7,8,3',4',5',6' (**25**) in *Distemonanthus benthamianus*[147] to mention only a few.

PRENYLATION

Generally, most flavonoid compounds are *C*-prenylated, whereas *O*-prenylation is rare. *C*-Prenylation takes place most frequently at positions 6/8 as well as 3'/5', and is usually represented by the 3,3-dimethylallyl chain, although 1,1-dimethylallyl, geranyl, lavandulyl, and farnesyl chains are also known, and will be collectively referred to as prenylated compounds. Flavonoid prenylation is one of the recently recognized conjugation reactions that was thought to be associated with isoflavonoids and pterocarpans of the Leguminosae.[85,86] However, recent surveys list more than 300 prenylated isoflavonoids including pterocarpans,[148] as well as 700 prenylated compounds belonging to other classes of flavonoids,[149] especially flavanones> flavones> chalcones> flavonols> dihydroflavonols> dihydrochalcones. Even some flavans and 1,3-diphenylpropanes are also prenylated.[149] This indicates a common occurrence but not necessarily a wide distribution, since prenylated flavonoids accumulate in a relatively small number of genera belonging to unrelated families.[149]

Enzymatic prenylation is catalyzed by prenyltransferases (PTs) that utilize dimethylallyl pyrophosphate (DMAPP) or an appropriate alkyl analog as the prenyl donor. In contrast with the cytosolic PTs involved in terpenoid biosynthesis, *e.g.*, geranyl-, farnesyl-, and geranylgeranyl pyrophosphate synthases,[150] aromatic PTs are membrane associated and require a divalent cation, preferably Mn^{2+} or Mg^{2+} for catalytic activity. The enzymatic prenylation of a variety of isoflavonoids includes the 6-prenylation of the isoflavones, genistein and 2'-hydroxygenistein, in white lupin,[151] 10-prenylation of 3,9-dihydroxypterocarpan in *Phaseolus vulgaris*,[152] 2- and 4-prenylation of 3,6a,9-trihydroxypterocarpan in *Glycine max*,[153,154] all of which were elicited with a yeast extract[152] or with a fungal cell wall elicitor.[154] In contrast, prenylation of genistein and 2'-hydroxygenistein at the 6-, 8-, and 3'-positions is catalyzed by three distinct, substrate-specific PTs in white lupin radicles and cell cultures,[155] which constitutively synthesize a variety of mono- and diprenylated isoflavones.[156] These PT activities were separated from a DOM (1-*O*-*n*-dodecyl-(α-D-glucopyranosyl)-β-D-pyranoside)-solubilized microsomal pellet by chromatography on a Fractogel TMAE-EMD 650(S) ion exchange column.[157] Other flavonoid-specific PT activities include the kaempferol 8-PT in *Epimedium diphyllum* cell cultures, which also accepts apigenin, luteolin, and quercetin as substrates,[158] and naringenin 8-PT in *Sophora flavescens* cell cutures, which catalyzed the 6-prenylation and 8-lavandulylation of 2'-hydroxynaringenin.[159] This substrate specificity indicates that enzymatic prenylation is a later reaction in flavonoid biosynthesis, although the position of prenylation in the sequence of other substitution reactions, *e.g.*, methylation, glycosylation, and/or malonylation remains to be determined.

The fact that flavonoid PTs are membrane associated enzymes poses an inherent difficulty in their solubilization and purification to near homogeneity for subsequent cloning of their genes. As a result, in comparison with other flavonoid enzymes, the cloning of a flavonoid PT has yet to be reported. Recently, however, a cDNA encoding a membrane-associated, light-inhibited geranyl diphosphate:4-hydroxybenzoate 3-geranyltransferase was isolated and characterized from *Lithospermum erythrorhizon* root cell cultures, through nested PCR techniques using the conserved amino acid sequences among polyprenyltransferases for ubiquinone biosynthesis.[160] This enzyme is essential for the biosynthesis of the napthoquinone, shikonin, an antimicrobial metabolite of the plant. Being the first reported cloning of a plant PT that transfers a prenyl chain to an aromatic substrate, this heterologous probe should enable the cloning of other flavonoid-specific PTs and the study of their phylogenetic relationships.

There is a growing consensus that most preformed flavonoid compounds are considered constitutive antimicrobial substances, recently termed phytoanticipins[161] that include prohibitins, inhibitins, or postinhibitins, in contrast with the inducible phytoalexins.[162] The presence of prenyl groups increases the lipophilicity and hence, the antifungal and anti-insect properties of most flavonoids,[149] especially the isoflavonoids.[8, 148] However, several examples[148,149] seem to indicate that antifungal activity may be due to the position of prenylation rather than the number of prenyl groups on the flavonoid ring, and cyclization of the prenyl side chain may reduce or abolish biological activity. The biological activities of the different classes of prenylated flavonoids have been reviewed in several articles.[1,8,86,148,149]

Of the different flavonoid substitutions, prenylation represents one of the most diversified structural variations. The rich variety and structural diversity of prenylated flavonoids are dependent on (a) the type of flavonoid class and the oxygenation pattern of the aromatic carbons, (b) the type (*C*- or *O*-) and position of prenylation, (c) the presence of other substituents, *e.g.*, methyl groups and/or glycosyl/malonyl residues, and (d) modification of the prenyl side chain by oxidation, reduction, dehydration, and/or cyclization. Cyclization of the isoprenoid side chain is catalyzed by a membrane-assoiated, cytochrome P450-dependent prenylcyclase,[163] which contrasts with the cytosolic, non-heme iron protein involved in the cyclization of the rotenoid, deguelin.[164] The following is a brief overview of the structural variations among prenylated flavonoids; a more complete account including many complex/exotic structures appears in some recent reviews.[8,86,148,149]

Flavonoid Prenylation Patterns

Chalcones and Dihydrocalcones

The majority of chalcones are monoprenylated with a few di-/triprenyl derivatives, all of which involve positions 3'>5'>5>3>2. About one-third of the

prenylated chalcones are monomethylated at positions 2'>4'>6'>4. Although they possess characteristics similar to prenylated chalcones, the dihydrochalcone derivatives are much less abundant.[149]

Flavanones and Flavones

In spite of the central role of flavanones as common intermediates in the biosynthesis of other flavonoid classes, prenylated flavanones represent the most abundant class of compounds (ca. 54% of the total).[149] The majority are di- and triprenylated derivatives with an appreciable number of geranyl/lavandulyl and farnesyl residues at positions 8>>6>5'=3'>2'>7, and with dimethylchromeno or furano derivatives at positions 7,8>6,7>3',4'>4',5'. About one-third are *O*-methylated (with one to four methyl groups), mostly at positions 7 and/or 4'>6>3'. Some exotic flavanones carry multiple prenyl groups and a single methoxyl group, or multiple methoxyl groups and one prenyl group. This presents a challenge to the biochemist as to the regiospecificity and sequential order of the enzymes involved in biosynthesis. Flavones are mostly prenylated at positions 6>8>7,8>6,7>3; about 20% of which are di- to penta-*O*-methylated at positions 7>5>4'>3'. The frequent 5-*O*-methylation seems to prevent chelation with the adjacent carbonyl group, resulting in stability of the prenylated flavone.

Isoflavonoids

Almost half of the isoflavonoids identified from the Leguminosae, in which prenylated isoflavones constitute >50% of the total (pterocarpanoids, rotenoids, isoflavanones, isoflavans, coumestans, 3-arylcoumarins, and coumaronochromones), exhibit a high degree of structural diversity and complexity of prenylation.[8,86,148] More than half of the isoflavones are monoprenylated, whereas 40% are diprenylated, and very few are triprenyl derivatives, with prenylation at positions 6>8>3'>>5'>6'>2' and *O*-methylation at 4'>7=6=2'=3'>5'=5>8. A number of novel isoflavonoid structures with rare skeletons, as well as complex isoflavone *O*- and *C*-glycosides have recently been reported.[8,148]

Dihydroflavonols and Flavonols

The great majority of prenylated dihydroflavonols are of the *2R,3R* configuration, and only a few have the *2R,3S* cofiguration, with prenylation at positions 8>6>6,7>7,8>3' and rare substitutions at other B-ring positions.[149] About one-third of the compounds are *O*-methylated at positions 5>4'>7=3. Prenylated flavonols, on the other hand, have a significant proportion of furano- and dimethylchromeno side attachments, but with a few di- and triprenyl derivatives, with prenylation at positions 8>6>4'>3'; however, alkylation at both A and B rings is

relatively rare. The majority of prenylated flavonols are mono- to tetra-*O*-methylated at positions 3>>8>6=5>3'>4'>7. Such a high frequency of *O*-methylation increases the lipophilicity of these metabolites and enhances their biological activity.[149] Although not very common, the majority of prenylated flavonol glycosides are typically 8-dimethylallyl flavonols with sugar residues and methyl groups at positions 3, 7 and/or 4', thus maintaining a balance between their hydrophilic and lipophilic natures.[150] More than one-half of the compounds are 3-*O*-diglycosides with Rha>>Glc>Xyl>Gal, whereas the remaining are 7-*O*-monoglucosides with the exception of 4'-O-rhamnoside.[149]

1,3-Diphenylpropanes

Although relatively small in number, these compounds are characterized by a high level of prenylation, including four di-, four tri-, and one tetraprenyl derivatives, and a low level of *O*-methylation with only two 2'-*O*-methylated compounds.[149] Whether enzymic prenylation occurs prior to, or after, assembly of the diphenylpropane structure has yet to be determined. Examples of the diversity of prenylation among different flavonoid classes are shown in **(26-33)**.

PERSPECTIVES

Plants represent a precious renewable resource and an important 'treasure chest' for the rich variety and enormous diversity of flavonoid compounds. It is evident that flavonoids exhibit a broad range of functions, most of which are dependent on the presence of various modifications to the basic carbon skeleton. Several classes of enzymes are responsible for the wide array of metabolites produced. Their activities are not necessarily mutually exclusive, although they may act in a sequential manner, with one modification determining future substitution events. Nevertheless, there are numerous flavonoid compounds that have been isolated whose synthesis remains unclear. Efforts directed towards the elucidation of the biosynthesis and physiology of these novel compounds should yield valuable information that can be applied in various systems.

It is of utmost importance that we understand how and why a compound is synthesized, in order to facilitate any future engineering efforts to modify flavonoid metabolism. With the aim of enhancing the horticultural and/or nutritional value of plants,[6,165] metabolic engineering techniques are presently being used to improve crop plants by modifying their flavonoid content. By increasing the amount of useful flavonoids present in a particular crop, or altering the types of flavonoids produced, its nutritional benefit may be elevated and, at the same time, plants with enhanced phytoalexin production may become less susceptible to disease. Furthermore, forage crops may be improved by altering the amount of condensed

tannins present in their tissues. Significant advances have also been made with respect to the modification of flower color, through a better understanding of the factors involved in co-pigmentation, vacuolar pH regulatory mechanisms, and co-factors required for substitution reactions.

Recently, there has been a marked shift towards genomics-based projects, making model plants the focus of research in the field of secondary metabolite production. This work will be invaluable in elucidating the regulation and functions of various flavonoid biosynthetic genes and their products at the organismal level. Nevertheless, non-model plants are likewise essential, since they can be used to isolate compounds with rare or more elaborate structures, in addition to providing insights as to what novel effects these metabolites may exert on the organism. Therefore, it will only be through combined efforts in phytochemistry, biochemistry, and molecular biology that sufficient information will be gained to effectively alter flavonoid metabolism.

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Chapter Two

STRUCTURAL, FUNCTIONAL, AND EVOLUTIONARY BASIS FOR METHYLATION OF PLANT SMALL MOLECULES

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INTRODUCTION

Plants produce over 100,000 small molecules, derived from phenylpropanoid, isoprenoid, and alkaloid biosynthetic pathways. This rich chemical diversity is the result of a rapidly diversifying and ongoing evolutionary process. Recent advances in the molecular biology of plants, particularly in the area of large scale genomics, are revealing how enzymes of secondary metabolism arise through processes of mutation and gene duplication, leading to continued elaboration of new chemical structures that will be selected for as they impart an adaptive advantage for the plant.[1] Many of these compounds act as chemical cues for plants during their on-going interactions with physical and biotic factors in their environments. In addition, plant metabolites have positive and negative impacts on human and animal health and nutrition. These diverse roles in plant and animal physiology provide impetus for molecular approaches to crop improvement based upon manipulation of natural product profiles. With the advent of wide scale genomic sequencing, the necessity of gene product characterization increases concomitantly. Structural biology provides an important tool for the detailed characterization of proteins at the atomic level. This level of functional understanding can lead to a more complete appreciation of complex biosynthetic pathways by elucidating the mechanisms of individual biosynthetic reactions.

Plant small molecule methyltransferases (MTs) constitute a large and important class of enzymes critical to many metabolic pathways. Most MTs methylate hydroxyl and carboxyl moieties, referred to as O-methyltransferases (OMTs), and occasionally target nitrogen and sulfur atoms of small molecules.[2] Often, the chemical characteristics of plant small molecules are tuned by the methylation patterns of the oxygen containing groups. Thus, the large number of reactive oxygen moieties on related carbon scaffolds necessitates many OMTs, which are capable of recognizing not only a specific carbon skeleton, but also unique positions on multiply hydroxylated or carboxylated compounds. The number of putative plant small molecule MTs listed in sequence databases is rapidly increasing due to depositions from a number of EST and genomic sequencing projects.[3,4] The MTs discussed in this proposal are divided into three different families based on sequence alignments (Fig. 2.1) and structural studies (Fig. 2.2).

Type 1 MTs, currently exclusive for oxygen atoms (OMTs), methylate hydroxyl moieties of phenylpropanoid-based compounds (Fig. 2.3). The phenylpropanoid scaffold is used as a building block for many other types of compounds in the plant. Modification of this basic unit by multiple condensation reactions yields chalcone, flavonoid, isoflavonoid, and pterocarpan skeletons, for example. Flavonoids are ubiquitous in higher plants, where they function as UV protectants,[5] defense compounds,[6] and stimulators of beneficial mutualistic interactions with microorganisms, insects, and other organisms.[7] Isoflavonoid natural products are limited primarily to leguminous plants, where they function as pre-

formed or inducible antimicrobial (phytoalexins) or anti-insect compounds, as inducers of the nodulation genes of symbiotic *Rhizobium* bacteria, and as allelopathic agents.[8] Additionally, both flavonoids and isoflavonoids have received a considerable amount of attention in view of reports implicating them as dietary components that may protect against cardiovascular disease, cancer, and osteoporosis.[9-13]

Type 2 MTs, also currently understood to function exclusively as OMTs, are found in all lignin-producing plants. This family is specific for coenzyme A derivatized phenylpropanoid compounds and appears to be less diverse in sequence and gene number than the type 1 family of MTs (Fig. 2.3). Based on database searches, type 2 OMTs consist of caffeoyl and feruloyl coenzyme-A specific OMTs (CCoAOMTs). The most closely related enzymes to plant CCoAOMTs are the mammalian small molecule methyltransferases, including catechol OMT and bacterial MTs involved in macrolide biosynthesis.

The third family of plant MTs consists mostly of enzymes that convert carboxylic acids to methyl ester derivatives (Fig. 2.3). Salicylic acid carboxyl methyltransferase (SAMT) from *Clarkia breweri*[14] provides the prototypical structure for a type 3 MT family member and includes benzoic acid carboxyl methyltransferase (BAMT) from snapdragons.[15] Database searches with the *Clarkia* SAMT amino acid sequence yields other MTs and numerous putative proteins, most annotated as jasmonic acid (JMT) or SAMT-like methyltransferases. *Arabidopsis thaliana* contains 24 genes belonging to this growing family of plant MTs,[16] including a *bona fide* JMT.[17] In addition to carboxylate specific MTs, SAMT bears sequence similarity to the alkaloid N-methyltransferases (NMT) involved in caffeine biosynthesis. Thus, type 3 MTs constitute a much larger structural family then originally anticipated, containing not only carboxyl group specific OMTs but also some alkaloid NMTs. Recently, the type 3 MT family of plant small molecule methyltransferases has been termed the SABATH family.[16]

In this chapter, we summarize recent structural work from our laboratory, which has led to a number of critical insights into the mechanism of action of each MT family as well as insight into the molecular basis for substrate diversification within and between families. However, several important questions remain to be answered. What are the architectural determinants for the binding of physiologically important substrates for which we currently lack a clear understanding? Can predictive homology models be constructed from the current set of structures, or do we need to further define the architecture of related enzymes with vastly different substrate specificities using the tools available in structural biology (x-ray crystallography and nuclear magnetic resonance spectroscopy)? Can the structural studies completed and planned be used to greatly increase the speed of gene annotations and functional characterization of new gene products? Finally, can the structural elucidations be used to create mutant enzymes capable of producing novel products?

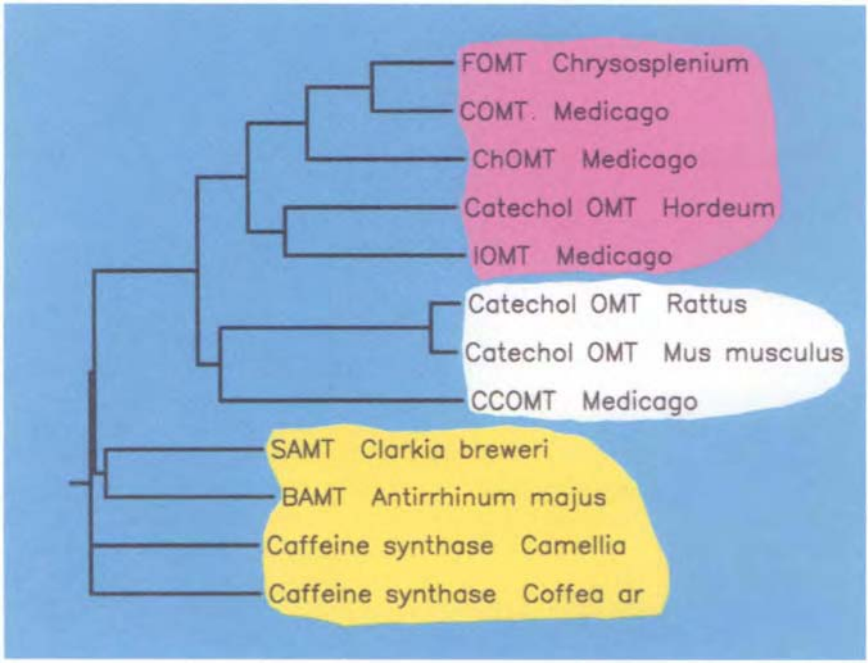

Figure 2.1: Sequence relationships in the form of a phylogentic tree of representative type 1, 2, and 3 MTs. The genus of each enzyme is listed. Type 1 (pink) family members include COMT, ChOMT, plant catechol OMT, and IOMT. Type 2 (light grey) includes catechol OMT from mammals and CCoAOMT (or CCOMT). Type 3 or SABATH (yellow) family members are predominantly carboxyl-directed MTs such as SAMT, BAMT, and JMT, but also include the NMTs involved in the biosynthesis of caffeine.

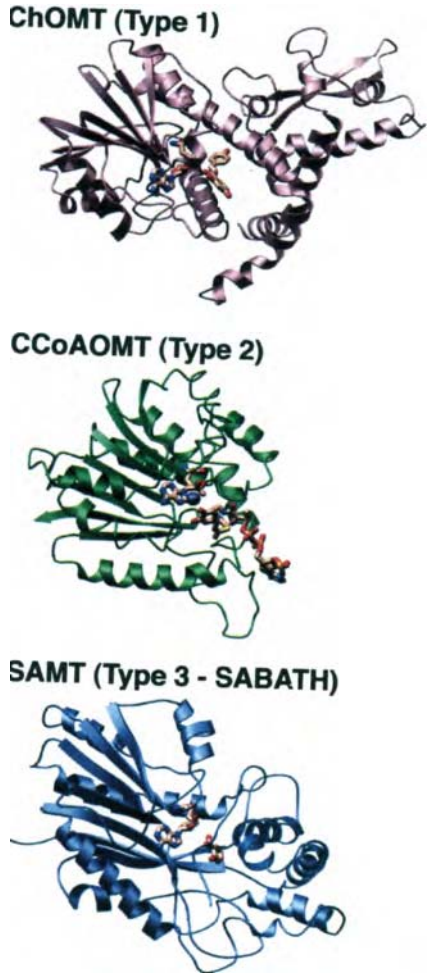

Figure 2.2: Overall architectural comparisons of type 1, 2, and 3 (SABATH) plant MTs discussed in this chapter and rendered as backbone ribbon diagrams.

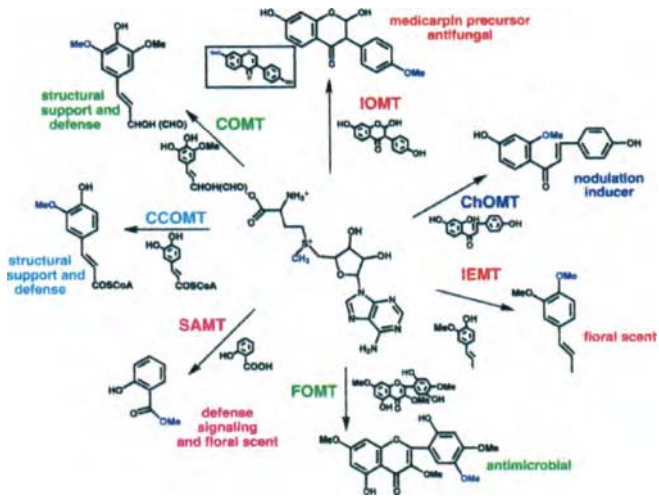

Figure 2.3: Summary of plant MTs and their associated reactions being examined structurally in our laboratory. SAM is shown in the center and serves as the universal methyl donor for all plant MTs examined to date.

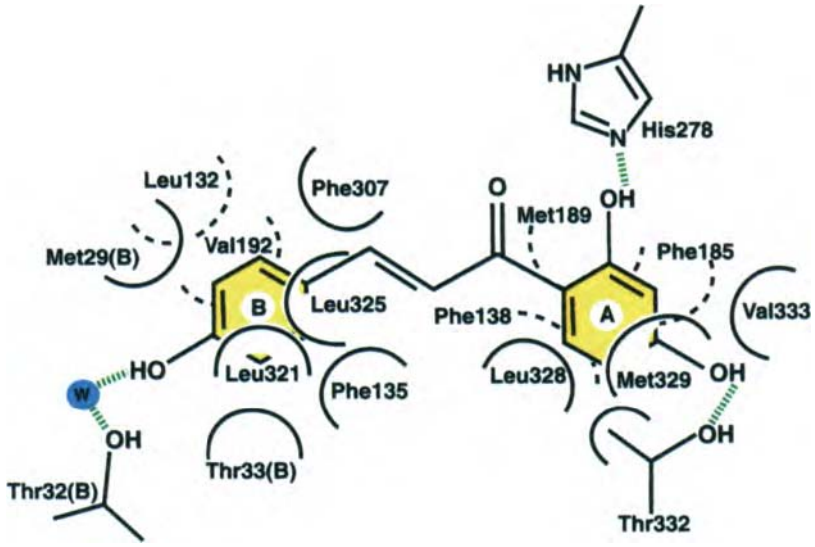

Figure 2.4: Schematic representation of the chalcone binding site of ChOMT. Hydrogen bonds are depicted as green dashed lines. Spatial orientation of residues is approximate with solid lines representing residues in the foreground and dashed lines representing residues in the background.

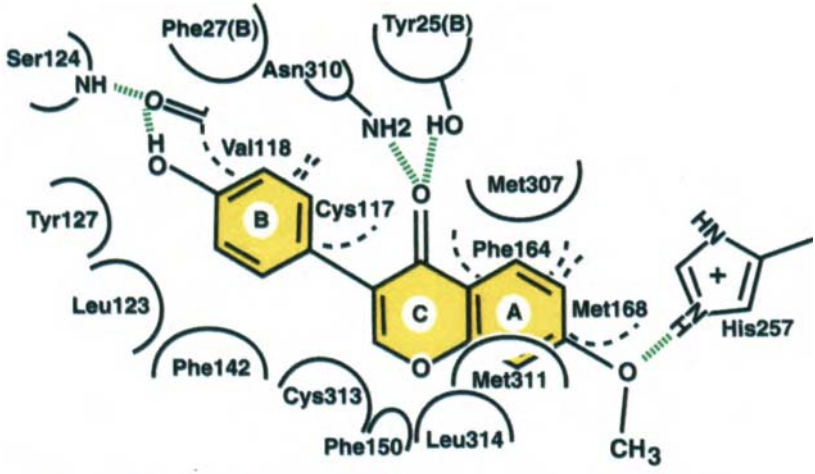

Figure 2.5: See following page.

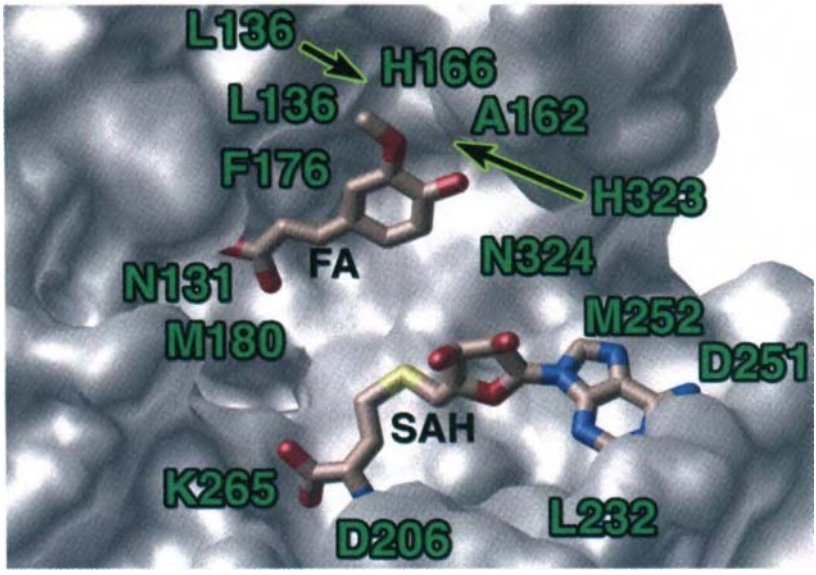

Figure 2.6: See following page.

Figure 2.5: Schematic representation of the complete isoflavone binding site of IOMT. Hydrogen bonds are depicted as green dashed lines. Spatial orientation of residues is approximate with solid lines representing residues in the foreground and dashed lines representing residues in the background

Figure 2.6: Close-up view of the COMT active site. Surface representation of the active site cavity for the COMT – SAH – ferulate (FA) complex illustrating the complementary shape and size to FA and SAH.

ARCHITECTURE OF TYPE 1 O-METHYLTRANSFERASES

Chalcone O-Methyltransferase (ChOMT)

ChOMT methylates the 2'-OH of 2',4,4'-trihydroxychalcone (isoliquiritigenin), converting it to 4,4'-dihydroxy-2'-methoxychalcone.[18,19] Among the many compounds released from the roots of alfalfa, 4,4'-dihydroxy-2'-methoxychalcone acts as the most efficient transcriptional activator of *nod* genes, activating *nodABC* through interaction with the transcriptional regulators *nodD1* and *nodD2* of *Rhizobium meliloti*.[19] Additionally, ChOMT-mediated methylation of isoliquiritigenin prevents the chalcone isomerase (CHI) catalyzed cyclization of isoliquiritigenin to the flavanone liquiritigenin (7,4'-dihydroxyflavanone).[20] Methylation of the 2'-OH of chalcone arrests biosynthetic flux through subsequent phenylpropanoid biosynthetic pathways. Thus, ChOMT acts as a branch-point enzyme, preventing the conversion of chalcone into diverse natural products including anthocyanins, flavonols, pterocarpans, and isoflavones.

Unlike most structurally characterized methyltransferases of non-plant origin that are monomeric, ChOMT forms a symmetric homodimer.[21] Dimerization appears to be critical for activity *in vitro* as well as *in vivo*. The presence of a dimerization interface appears to be common to plant OMTs, and in this family of OMTs this homotypic protein-protein interface intimately contributes to substrate binding. Because of the broad structural diversity of phenylpropanoid compounds, ChOMT, like many plant OMTs, possesses highly selective substrate and positional specificity. Efficient substrate discrimination and binding is achieved through shape

selectivity dictated by van der Waals interactions and by specific hydrogen bonding patterns. The isoliquiritigenin substrate adopts two conformations within the active site *via* a ~180° rotation around the carbonyl carbon, resulting in two distinct binding modes for the B-ring of the substrate. The position of the A-ring, which presents the 2'-OH group to SAM for methylation, is conserved in both conformers. The A-ring is bound by the thioether moieties of Met 329 and Met 189 and maintains a preferred edge to face aromatic interaction with Phe 185. Thr 332 and the 4'-OH of the substrate are within hydrogen bonding distance, which secures the substrate within the active site and most likely ensures that the A-ring 2'-OH is properly positioned for methylation. The back wall of the active site consists of residues Met 29, Thr 32, and Thr 33 donated from the partner monomer (Fig. 2.4).[21]

Isoflavone O-Methyltransferase (IOMT)

IOMT acts on isoflavone compounds *in vitro* and isoflavanone compounds *in vivo*. IOMT is essential for the biosynthesis of medicarpin, the major phytoalexin of alfalfa.[22] *In vivo* studies demonstrate that IOMT is necessary for the formation of 7-hydroxy-4'-methoxyisoflavone (formononetin). *In vitro* assays using 4',7-dihydroxyisoflavone (daidzein) as substrate and *in vivo* studies conducted in the absence of fungal elicitation of IOMT-overexpressing plants yield 4'-hydroxy-7-methoxyisoflavone (isoformononetin).[23] This compound is rarely found in plants and has no known biological role in plant physiology. However, when elicited with $CuCl_2$ or infection with *Phoma medicaginis*, IOMT-overexpressing plants accumulate the 4'-O-methylated isoflavonoid formononetin and the downstream phytoalexin derived from it, medicarpin. IOMT exhibits the same tertiary structure as ChOMT, consisting of a C-terminal catalytic domain responsible for SAM and substrate binding and methylation and a smaller N-terminal domain involved primarily in dimerization.

Tyr 25, Phe 27, and Ile 28 insert into the back wall of the catalytic domain of the dyad related monomer. This type of dimerization interface appears to be typical of type 1 plant OMTs and is a defining element of the sub-family. Due to the lack of aqueous stability exhibited by the isoflavanone substrate, 2,7,4'-trihydroxyisoflavanone, the isoflavone, daidzein, was substituted in crystallization experiments because IOMT exhibits considerable activity towards this compound. Co-crystallization of IOMT with SAM and daidzein resulted in the product complex of SAH and isoformononetin. Extensive aromatic-aromatic interactions are present between Phe 150, Phe 164, Phe 142, Tyr 127, and the A-ring of the isoflavone product, isoformononetin. As in ChOMT, Met 168 and Met 311 constrain the A-ring and help position the 7-OH group for methylation. Asn 310 and Tyr 25 (B), from the dyad related monomer, form a hydrogen-bonding network with the carbonyl oxygen of the C-ring of daidzein. The backbone amide nitrogen of Ser 124 and the carbonyl oxygen of Val 118 hydrogen bond with the 4'-OH of the B-ring, maintaining the

isoflavone in a single orientation. Tyr 25, Phe 27, and Ile 28 of the symmetrically arranged monomer form the back wall of the active site and have important consequences for determining substrate specificity (Fig. 2.5).[21]

Based both upon the structures of ChOMT and IOMT and sequence alignments with the large family of plant OMTs, methylation in the type 1 family of plant MTs most likely proceeds via base-assisted deprotonation of the targetted hydroxyl group, followed by a nucleophilic attack of the newly generated phenolate anion of the substrate on the reactive methyl group of SAM. For example, in IOMT, His 257 serves as the base responsible for deprotonation of the 7-hydroxyl group on the A-ring of daidzein. Moreover, Asp 288 and Glu 318 sterically constrain His 257 and position the Nδ. Mutations of His 257 to leucine, isoleucine, glutamine, and aspartate eliminated methyltransferase activity towards daidzein.[21]

Caffeic Acid / 5-Hydroxyferulic Acid 3/5-O-Methyltransferase (COMT)

Lignin is a principal structural component of cell walls in most vascular terrestrial plants. Moreover, after cellulose, lignin is the second most abundant plant polymer. The lignin polymer is composed of phenylpropane units oxidatively coupled through carbon-oxygen and carbon-carbon linkages. This natural polymer also functions as a genetically inducible physical barrier in response to microbial attack.[24-28] In angiosperms, lignin is composed of two major monomeric phenolic constituents, namely guaiacyl (G) and syringyl (S) units. The G unit is singly methoxylated at the 3-OH position, whereas the S subunit is methoxylated at the 3-OH and 5-OH positions. The ratio of S/G subunits dictates the level of lignin condensation by allowing for different types of polymeric linkages. Increased G content leads to a more highly condensed lignin composed of a greater proportion of biphenyl and other carbon-carbon linkages, whereas S subunits are commonly linked through more labile ethereal bonds.[29-31]

The structural determination of one of the critical methyltransferase enzymes in this pathway, namely COMT, not only provides a structural basis for understanding substrate discrimination, but will also enable the introduction of targeted mutations into these enzymes to modulate MT activity and help identify *in vivo* metabolites for lignin biosynthesis. COMT from alfalfa (*Medicago sativa*) is an S-adenosyl-L-methionine (SAM)-dependent, multifunctional MT that methylates 3- and 5-hydroxylated phenylpropanoids.[32] COMT-type enzymes are widespread throughout the plant kingdom and found in all lignin-producing plants. Unlike the two previously discussed enzymes, COMT demonstrates a greater degree of substrate promiscuity and activity at different hydroxyl sites, as exhibited by its high activity towards 3- and 5-hydroxylated phenylpropanoids. *In vitro* studies have shown the enzyme methylates caffeic acid, 5-hydroxyferulic acid, caffeoyl aldehyde, caffeoyl alcohol, 5-hydroxyconiferyl aldehyde, and 5-hydroxy-coniferyl alcohol

yielding the methoxy phenylether and S-adenosyl-L-homocysteine (SAH) as products.[33]

Ferulic acid (ferulate) and 5-hydroxyconiferaldehyde bind in a similar fashion to the active sites in the two complexes that were characterized crystallographically in our lab. In both cases the hydrophobic residues Phe 176, Met 130, Met 320, and Met 180 sequester the phenyl ring that presents the reactive hydroxyl group to SAM. Hydrogen bonding of His 166 with the 3-methoxy group of ferulic acid helps orient the substrate for preferential methylation of the 5-OH position. A slightly altered substrate orientation is observed for binding of 5-hydroxyconiferaldehyde. His 166 forms a hydrogen bond with the hydroxyl moiety at the 4-position of the substrate, and additional hydrogen bonds form between Asp 270 and Asn 324 and the 5-OH group. The orientation observed for 5-hydroxyconiferaldehyde presents the 5-OH moiety for methylation. Moreover, the methoxy groups of both ferulic acid and 5-hydroxyconiferaldehyde reside in a complementary hydrophobic methyl-binding pocket consisting of Leu 136, Phe 172, Phe 176, and Ala 162 (Fig. 2.6). This conserved pattern of favorable van der Waals interactions most likely explains the kinetic preferences of COMT for the 3-methoxyl-4,5-dihydroxyl-substituted substrates over the 3,4-dihydroxyl-substituted substrates. The propanoid tails of the potential COMT substrates include carboxylate, aldehyde, and alcohol functionalities. In order to accept different substrates, the active site must be spacious and versatile enough to allow the binding of these different molecules.[34]

The environment for binding the propanoid terminus consists of two water molecules and the hydrophilic residues His 183 and Asn 131. The water molecules mediate binding interactions between His 183, Asn 131, and the hydrophilic tail of the substrates. The same hydrogen bonding pattern and position of the substrate tail were observed for the aldehyde and carboxylate containing substrates. In addition to the hydrophilic residues, the propanoid tail is surrounded by a number of hydrophobic groups including Met 180, Met 130, Ile 316, and Ile 319. The structure and relative hydrophobicity of this propanoid tail-binding pocket suggests that selectivity for neutral aldehydes and alcohols over negatively charged carboxyl groups is conferred by this region of the COMT active site.[34]

ARCHITECTURE OF TYPE 2 O-METHYLTRANSFERASES

Caffeoyl / 5-Hydroxyferuloyl Coenzyme-A 3/5-O-Methyltransferase (CCoAOMT)

As briefly described above, proposed biosynthetic pathways to the monolignols follow a metabolic grid, with a number of parallel and redundant pathways for the synthesis of the major lignin components, namely guaiacyl and syringyl units. Currently, the methylation steps in the lignin biosynthetic pathway are believed to follow two main branches dependent on COMT and CCoAOMT,

respectively. CCoAOMT methylates caffeoyl and 5-hydroxyferuloyl CoA thioesters with an *in vitro* kinetic preference for caffeoyl CoA.[33,35-37] The CoA thioester is cleaved and reduced in successive steps to form the aldehyde and alcohol substituted monolignols.

Primary sequence analysis of CCoAOMT and comparisons with other plant OMTs demonstrates that the enzyme belongs to a distinct family of plant MTs. This second family includes plant OMTs that exhibit a high degree of specificity for CoA esters, with sequence identity ranging from 50% to 90%. The closest group to this family, based on sequence identity, is the group of animal catechol OMTs. The rat catechol OMT (19% sequence identity with CCoAOMT) has been structurally characterized[38] and used to model the structure of CCoAOMT.[39]

Another group of MTs closely related to plant CCoAOMTs includes enzymes responsible for the methylation of macrolides, such as macrolide 4-O-methyltransferase (Ma4OMT, 37% sequence identity with alfalfa CCoAOMT) from *Streptomyces mycarofaciens*[40] and macrolide 8-O-methyltransferase (Ma8OMT, 10% sequence identity with alfalfa CCoAOMT) from *Streptomyces glaucescens*.[41] Macrolides are polyketides produced in bacteria by modular polyketide synthases and decorated by post-polyketide tailoring enzymes, including Ma4OMT and Ma8OMT, giving rise to a large family of natural compounds with antibiotic properties.[42] Genes coding for Ma4OMT (accession number Q00719) and Ma8OMT (accession number P39896) are members of gene clusters involved in production and alteration of midecamycin and tetracenomycin C, two important macrolide compounds.

CCoAOMT, unlike type 1 MTs, consists of a single domain, comprised primarily of the Rossmann fold (Fig. 2.7). This MT lacks the N-terminal dimerization domain seen in ChOMT, IOMT, and COMT, lending further evidence for the unique designation of CCoAOMT into a second structural family (Fig. 2.3). Alfalfa CCoAOMT is a 28 kDa protein with 247 amino acids that forms a dimer in solution.[43] The crystallographic structure obtained at 2.45 Å resolution (2.65 Å with substrate) reveals the dimer observed in solution. Due to the lack of the N-terminal dimerization domain present in previously characterized plant OMTs, the interaction between monomers is carried out in a completely different way, involving residues 26-40, 66-78, and 212-226 as mostly hydrophobic interactions. The first 20 amino acid residues, assumed to undergo conformational changes upon substrate binding, are not observed in the calculated electron density maps. The surface residues Arg 206 and Lys 21 interact with the negatively charged 3'-phosphate group of the adenosine 3',5'-diphosphate moiety of CoA. Much of the pantetheine phosphate is exposed to solvent and, due to the number of degrees of freedom, is not well defined in the refined crystal structure.

Evidence for Metal Ion-Dependent Methylation

A calcium atom is observed in the active site, surrounded by an octahedral coordination shell. The side chain oxygens of Thr 63, Glu 67, Asp 163, Asp 189, and Asn 190 are involved in the chelation of this calcium atom, completed by the 3-hydroxyl group of caffeoyl CoA or 5-hydroxyferuloyl CoA and a water molecule. This calcium atom substitutes the Mg^{2+} atom expected at this position, and is present in the catechol OMT structure with the same coordination geometry.[2,38] In CCoAOMT, Mg^{2+} mediates the deprotonation of the caffeoyl 3-hydroxyl group and maintains the 3-hydroxyl group in close proximity to the reactive methyl group of SAM (~3Å), suitably positioned for facile transmethylation to occur (Fig. 2.8).

Residues involved in phenolic substrate recognition reside mostly on the C terminal half of CCoAOMT. This region encompasses Met 61, Asp 163, Asn 190 plus a set of aromatic residues including Tyr 208, Tyr 212, and Trp 193 (Fig. 2.8). Each substrate and cofactor interact with only one monomer of the CCoAOMT dimer, unlike the arrangement seen in type 1 MTs.[21,34]

ARCHITECTURE OF TYPE 3 (SABATH) CARBOXYL METHYLTRANSFERASES

O-Methylation of Ionized Carboxyl Groups

Small molecule carboxylates are an important class of compounds that plants produce to attract pollinators,[44] defend against herbivory,[45-47] regulate the cell cycle,[48,49] and induce defense responses.[50-52] Often these compounds are esterified and volatilized. Plants utilize the emission of volatile esters as chemoattractants and signaling molecules. Classes of volatile compounds include the salicylates and jasmonates, which are ubiquitously distributed in the plant kingdom. Jasmonic acid, salicylic acid, and their methyl ester derivatives, are intimately involved in cell regulation and help control such developmental processes as seed germination, flower development, fruit development, and senescence.[14,17,53]

Salicylates and jasmonates have been well characterized in a number of plant species such as *Arabidopsis thaliana*,[17] *Nicotiana tabacum*,[54] and *Clarkia breweri*.[44,55] They are implicated in localized and systemic defense responses and in acquired disease resistance.[50,51] For example, upon pathogen challenge, plants increase salicylic acid biosynthesis and mount an initial defense response. This salicylic acid production can also trigger localized acquired resistance (LAR) to subsequent pathogenic attack, as well as induce systemic acquired resistance (SAR) in which pathogenic resistance is not restricted to the initial site of challenge, but is system wide.[54,56] Mounting evidence supports the role of methyl jasmonate as an interplant signaling molecule, which induces defense responses in uninfected

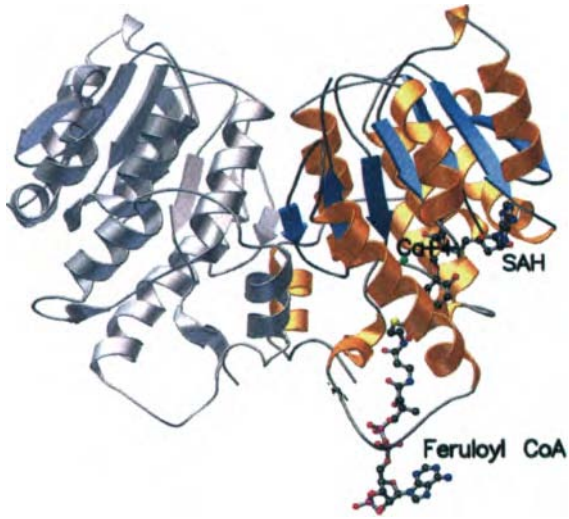

Figure 2.7: Ribbon diagram of the alfalfa CCoAOMT dimer, with one monomer colored and the other in grey. The SAH and feruloyl CoA molecules are depicted as bonds. The Ca^{2+} ion is in green.

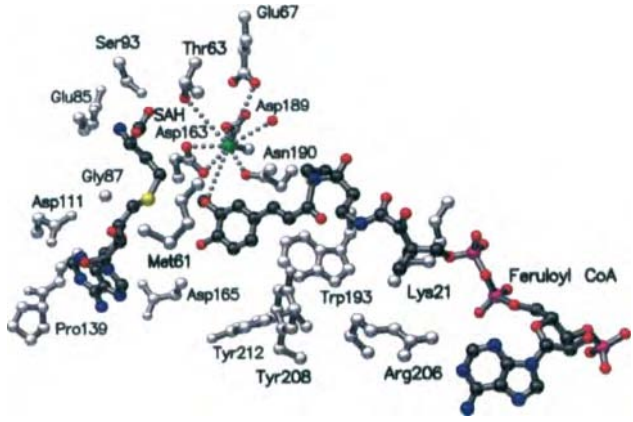

Figure 2.8: Active site close-up of CCoAOMT. Residues involved in Ca^{2+}, SAH, and substrate binding are displayed in light grey. Oxygen atoms of these residues participating in the coordination of Ca^{2+} ion are colored red.

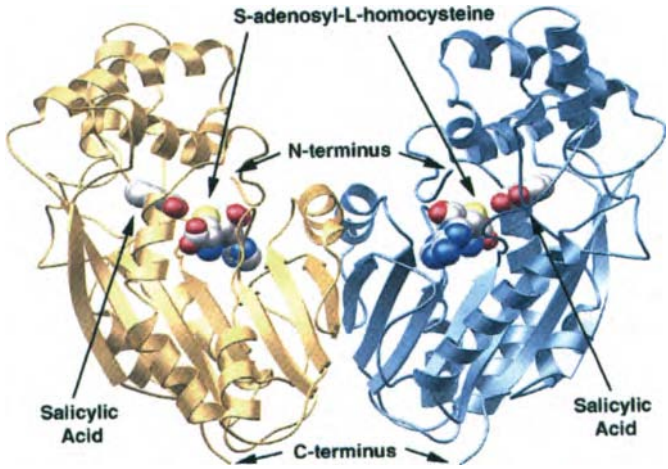

Figure 2.9: Ribbon diagram of the SAMT dimer. One monomer is blue, the other is gold. SAH and salicylic acid are depicted as CPK models with atoms color-coded by atom type.

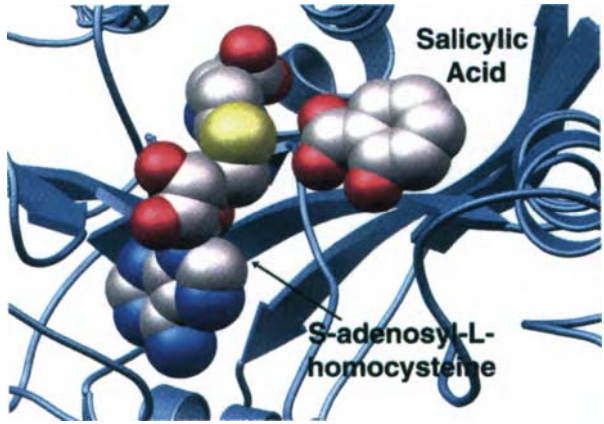

Figure 2.10: SAMT active site. The distance between salicylic acid and SAH in the crystal structure is 3 Å. Hydrogen bonding distances for Gln25, Trp151, and the carboxyl moiety of salicylic acid is 3.1 Å and 2.8 Å, respectively.

neighboring plants.[57,58] The volatile ester of salicylic acid, methylsalicylate, is hypothesized to act in a similar fashion as an air born signal, which triggers the defense response in uninfected organs of the plant or in adjacent, unchallenged plants.[59]

Salicylic Acid Carboxyl Methyltransferase (SAMT)

Originally isolated and characterized from the petals of *Clarkia breweri*, salicylic acid carboxyl methyltransferase (SAMT), converts salicylic acid to methylsalicylate.[14] Methysalicylate, a volatile ester, has a characteristic wintergreen scent. Apart from its putative role in defense, methylsalicylate is an important chemoattractant for moth-pollinated flowers and one of the main volatiles released from *Clarkia breweri* flowers.[44,55] SAMT is a member of the type 3 family of plant MTs. Similarly to the previously discussed families of methyltransferases, type 3 MTs are homodimeric proteins.

Because the three genes encoding the canonical members of this family - SAMT, BAMT, and JMT have only recently been characterized, the full extent of this family is not known. Database searches using the BLAST 2.0 program reveal a number of unknown gene products and putative SAMT -like enzymes as well as caffeine synthases belonging to the type 3 family of MT's. Thus, the family includes not only carboxyl methyltransferases but also some alkaloid N-methyltransferases.

The overall structure of the SAMT monomer consists of a single globular domain containing the extended beta sheet characteristic of the Rossmann fold and an N-terminal alpha-helical cap that forms the top of the active site. SAMT forms a homodimer in solution and this dimeric arrangement was preserved in the crystal structure (Fig. 2.9). The thioethers of Met 308 and Met 150 constrain and position salicylic acid for the transmethylation reaction. To favorably orient the substrate, hydrogen bonding interactions form between the carboxylate tail of salicylic acid and the indole nitrogen of Trp 151 and the amide nitrogen of Gln 25. In this structure, there are no amino acid residues or water molecules within hydrogen bonding distance of the 2-hydroxyl group (Fig. 2.10). Salicylic acid, a small aromatic molecule, utilizes a methionine sandwich motif to position the phenyl ring. This methionine rich active site is similar to previously characterized plant MT's, which also must position hydrophobic, aromatic substrates.

Catalysis appears to proceed simply by positioning an ionized carboxyl group within 3.0 Å of a reactive methyl group on SAM. The predominant driving force for transmethylation appears to be proximity rather then the active participation of SAMT active site residues in preparing the methyl acceptor atom.

SUMMARY AND FUTURE DIRECTIONS

The combined approach using structural and functional studies to understand biosynthesis provides an important basis for the study of related MT family members. An increased understanding of the molecular basis for MT substrate specificity and evolutionary divergence will permit one to rapidly assess the functional characteristics of novel but related enzymes recently discovered in numerous EST and genomic sequencing efforts. In addition, the successful introduction of novel specificities into existing enzymes by *in vitro* mutagenesis guided by a detailed knowledge of the three dimensional architecture of the MT active site, followed by plant transformation, should greatly increase the natural chemical repertoire available in plants. This form of structurally-guided metabolic engineering may enhance the disease preventing, nutritional, flavor, and fragrance properties of agronomically important crops.

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Chapter Three

REGULATION OF ANTHOCYANIN PIGMENTATION

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INTRODUCTION

While we find plant pigments intriguing and beautiful, they serve important roles in the ecophysiology of plants by attracting or deterring birds, insects, and animals. Many of the water-soluble reds and purples of flowers are variations of the same compounds – the anthocyanins (Greek: *anthos*, flower and *kyanos*, blue). First coined in 1835 by Marquat in a paper entitled "*Die Farben der Bluthen, eine chemisch-physiologische Abhandlung*", the name anthocyanin is used for the red, violet, and blue pigments of flowers, originally thought to be dehydration products of chlorophyll.[1] Today we know that anthocyanins are aromatic, glycosylated heterocyclic compounds that arise from the phenylpropanoid pathway. The swivel between the anthocyanin flavylium cation and the quinoidal base brings about a change in color towards the red at lower pH, and blue at higher pH.[2] The spectral shifts were first recorded by Robert Boyle in 1664 in his paper "*Experiments and Considerations Touching Colours*" where he describes the change from red to a perfect green of the blue "*Syrrup of Violets*" when exposed to "*Acid Liqour*" or a "*Solution of Potashes*".[1] However, specific chemistry of the compounds during these pH shifts has not been clearly established. Besides pH, many other factors including the interaction of anthocyanins with other phenolic compounds and the formation of metal complexes influence the pigmentation properties of anthocyanins.[3]

The most conspicuous function of vacuolar sequestered anthocyanins is to provide the bright red and blue colors that serve as attractants for pollination and seed dispersal. Anthocyanin pigments have appeared quite recently in evolution,[4] probably linked with the origin of the flowering plants. In contrast, other flavonoid compounds, already present in early photosynthetic plants, play more central roles in plant biology. These functions, extensively reviewed elsewhere,[5,6] include photoprotection, communication in plant-microbe interactions, hormone signaling, and male fertility.

The beautiful foliage color changes associated with the advent of autumn, the floral color shifts widely present in the angiosperms, and the effects that pathogens have on flower pigmentation are all phenomena that fill our days with colors and that highlight the complex mechanisms that control plant pigment formation. Thus, anthocyanin biosynthesis goes beyond just furnishing an ideal system for the genetic, biochemical, and molecular dissection of complex regulatory networks – it provides a means to understand the colorful world around us.

MAKING ANTHOCYANINS: ONE THREAD OF A COMPLEX WEB

The pigmentation provided by anthocyanins and other flavonoid-derived compounds has made this pathway a favorite for genetic studies since the early days of modern genetics. Today, mutants and the corresponding genes for more than 40

flavonoid biosynthetic enzymes or regulatory proteins are available, making it by far the best described plant metabolic pathway. Major contributions to the understanding of anthocyanin accumulation were made in model systems such as maize, petunia, snapdragon, and later complemented by *Arabidopsis.*[7-9]

Anthocyanins are flavonoids, which are a major offshoot of the highly branched phenylpropanoid pathway (Fig. 3.1, 3.2). The main amino acid precursor for phenylpropanoids is phenyalanine, although the monocot enzyme phenylalanine ammonia lyase (PAL) can also use tyrosine. Phenylalanine and tyrosine are derived from the shikimate acid/aerogenate pathway, which is feedback-regulated by the end products. Thus, apart from the internal multi-level regulation of the phenylpropanoid/flavonoid pathway, there exists a higher level of regulation of precursor and product pools that play a key role.[2] Experiments in *Arabidopsis* that used mutants at different steps in the pathway indicate that specific intermediates function as inducers for flavonoid accumulation.[10] How intermediates and final products in the pathway influence the activity of the anthocyanin regulators is not yet well understood.

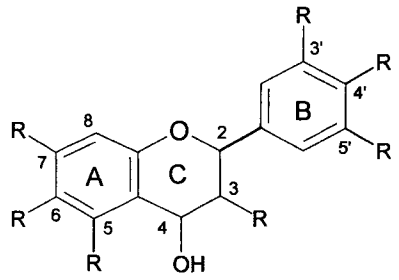


Figure 3.1: Basic carbon skeleton of a leucoanthocyanidin. The three rings are depicted as A, B and C. The 5 and 7 positions of the A ring are often hydroxylated. Rarely, a hydoxyl group occupies position 6. The B ring can be hydroxylated at the 3', 4' and 5' positions in various combinations. No hydroxylation at position 3 results in the less usual 3-deoxy flavonoids. Possible additional modifications at all the different positions have been extensively described elsewhere.[2,69]

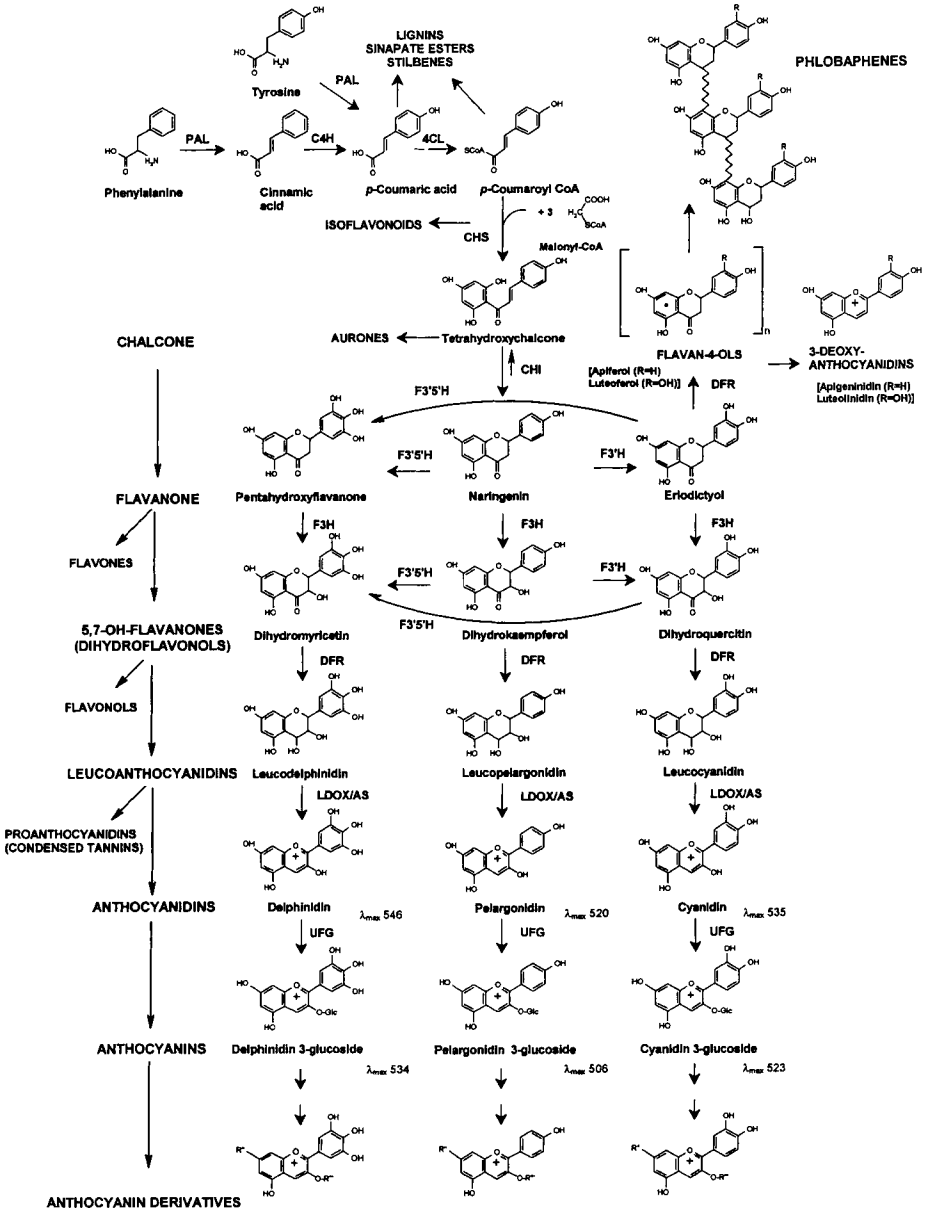

LIGNINS
SINAPATE ESTERS
STILBENES
PHLOBAPHENES
Tyrosine
PAL
Phenylalanine
PAL
C4H
4CL
Cinnamic acid
p-Coumaric acid
p-Coumaroyl CoA
+ 3
ISOFLAVONOIDS
CHS
Malonyl-CoA
CHALCONE
AURONES
Tetrahydroxychalcone
CHI
FLAVAN-4-OLS
[Apiferol (R=H)
Luteoferol (R=OH)]
DFR
3-DEOXY-
ANTHOCYANIDINS
[Apigeninidin (R=H)
Luteolinidin (R=OH)]
F3'5'H
F3'5'H
F3'H
FLAVANONE
Pentahydroxyflavanone
Naringenin
Eriodictyol
F3H
F3H
F3H
FLAVONES
F3'5'H
F3'H
5,7-OH-FLAVANONES
(DIHYDROFLAVONOLS)
F3'5'H
Dihydromyricetin
Dihydrokaempferol
Dihydroquercitin
DFR
DFR
DFR
FLAVONOLS
LEUCOANTHOCYANIDINS
Leucodelphinidin
Leucopelargonidin
Leucocyanidin
LDOX/AS
LDOX/AS
LDOX/AS
PROANTHOCYANIDINS
(CONDENSED TANNINS)
ANTHOCYANIDINS
Delphinidin
λmax 546
Pelargonidin
λmax 520
Cyanidin
λmax 535
UFG
UFG
UFG
ANTHOCYANINS
Delphinidin 3-glucoside
Pelargonidin 3-glucoside
Cyanidin 3-glucoside
λmax 534
λmax 506
λmax 523
ANTHOCYANIN DERIVATIVES

Figure 3.2: The core flavonoid pathway leading to the major groups of flavonoid pigments. The general phenypropanoid pathway converts phenylalanine and tyrosine into the precursor *p*-coumaryl CoA. The first committed step catalyzed by CHS condenses *p*-coumaryl-CoA with three molecules of acetyl-CoA derived from malonyl-CoA to form the fifteen carbon skeleton. We show here just the three most common anthocyanins, which upon modifications that include methylations, acylations, hydroxylations, and glycosylations yield a wide variety of derivatives. The abbreviations of the depicted enzymes are as follows: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3'H, flavonoid 3'hydroxylase; F5'H, flavonoid 5'hydroxylase; F3',5'H, flavonoid 3',5' hydroxylase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin dioxygenase/AS, anthocyanidin synthase; UFGT, UDP glucose:flavonoid 3-O-glucosyl transferase. λ_{max} values correspond to aqueous methanol HCl solutions. Steriochemistry has been omitted for the sake of simplicity.

The first step in the core flavonoid pathway leading to the anthocyanins (Fig. 3.2) involves the condensation of one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-CoA catalyzed by the homodimeric enzyme chalcone synthase (CHS), to form naringenin chalcone (4,2',4',6'-tetrahydroxychalcone). This first step commits the metabolic flux into the flavonoid pathway from which the chalcones, flavones, flavonols, flavan-diols, proanthocyanindins, and the anthocyanins arise (Fig. 3.2).[9] Chalcone isomerase (CHI) further cyclizes naringenin chalcone in a stereo-specific manner to from (*2S*)-naringenin (5,7,4' trihydroxyflavanone). This ring closure can occur spontaneously at a physiological pH >7.5 to yield a racemic mixture. However, (*2S*)-flavanone is the biologically active precursor utilized in the subsequent steps of flavonoid biosynthesis. While CHI is absolutely essential for anthocyanin formation in *Arabidopsis*,[11] anthocyanins can form in other systems without detectable CHI activity,[12,13] opening fundamental questions regarding the cellular function of CHI. Flavanone 3-hydroxylase (F3H), a soluble mono-oxygenase, catalyses the addition of the 3-OH group of the 'C' ring, which is later glycosylated by UDP-glucose:flavonoid 3-O-glucosyl transferase

(UFGT) in the terminal step of the synthesis of the stable anthocyanins.(Fig. 3.1, Fig. 3.2).

The flavonoid hydroxylases (F3'H, F3',5'H) and the phenylpropanoid enzyme cinnamate 4-hydroxylase (C4H) are cytochrome P-450s that are anchored to the cytoplasmic face of the ER.[14] F3'H and F3',5'H add hydroxyl groups to the 3' and/or 5' of the 'B' ring bringing about chemical and spectral diversity to the flavonoids. Dihydroflavonol 4-reductase (DFR) is the committed step to the production of the precursor of the colored compounds – the leucoanthocyanidins (Fig. 3.2). It is upon the reduction of the 4 keto of the 'C'-ring by DFR (Fig. 3.1) and its further reduction by leucoanthocyanidin dioxygenase/anthocyanidin synthase (LDOX/AS) that the de-localization of the electrons necessary for the formation of the planar flavylium ion is permitted.[2]

Interestingly, *Arabidopsis* CHS, CHI, and F3H form a multi-enzymatic complex with each other as well as with the next enzyme in the pathway, DFR.[15,16] There is a possibility that flux into different branches of the pathway could be directed through the formation of distinct complexes, with CHI playing more of a central structural role rather than a catalytic role, or maybe both and, thus, explain its essential nature in *Arabidopsis*.[16] However, the maize CHS, CHI, and DFR enzymes can complement the *Arabidopsis tt4*, *tt5*, and *tt3* mutations, respectively, despite the modest sequence identity between the monocot and dicot enzymes.[17] More studies are necessary to establish whether the formation of a "flavonoid metabolon" is a curiosity of *Arabidopsis* or a general property of this biosynthetic pathway.

While the CHS-CHI-F3H-DFR-AS enzymes form the core flavonoid biosynthetic pathway (Fig. 3.2), every intermediate compound in the pathway can be the subject of complex modifications that include hydroxylations, methylations, esterifications, and decorations with a number of sugar moieties. In addition, many of the core enzymes can utilize various substrates resulting in a pathway that is not linear, but rather a complex grid (Fig. 3.2).[2] The diverse forms of flavonoids or anthocyanins that accumulate in any plant under any given condition are the result of a combination of the biosynthetic enzymes being expressed together with their substrate specificity. Over the past few years, the structures of several flavonoid biosynthetic enzymes have been elucidated,[18-20] which opens up unlimited opportunities to understand structure-function relationships and to manipulate the pathway.

REGULATORS OF ANTHOCYANIN BIOSYNTHETIC GENES

In all plant systems investigated so far, controlling transcription of the flavonoid biosynthetic genes primarily carries out the regulation of anthocyanin accumulation. While many different types of regulatory proteins may participate in the transcriptional activation of flavonoid biosynthetic genes, best described and clearly conserved among the major groups of flowering plants is the participation of

R2R3 MYB and HLH transcription factors acting in concert (Table 3.1). More recently, proteins containing conserved WD40 repeats have been implicated in the regulation of flavonoid biosynthesis as well. Thus, it is by variations of the combination of MYB/HLH/WD factors that anthocyanin accumulation is controlled (Table 3.1).

Table 3.1: Known regulators of the different flavonoid pigment in various plants, classified according to the presence of distinctive domains (MYB, HLH, or WD40). Negative regulators are indicated by an asterisk. *

	MYB	HLH	WD40	Others
Anthocyanin biosynthesis				
***Zea mays* (Maize)**	**C1, PL**	**R, B, Sn, Lc IN1***	**PAC 1**	**A3**
***Petunia hybrida* (Petunia)**	**AN2, AN4**	**JAF13, AN1**	**AN11**	
***Antirrhinum majus* (Snapdragon)**	**ROSEA1, ROSEA2**	**DELILA**		
Arabidopsis thaliana	**PAP1, PAP2**		**TTG**	**ANL2 (HD)**
Perilla frutescens	**MYB-P1**	**MYC-RP/GP**		
Gerbera hybrida		**GMYC1**		
***Oryza sativa* (Rice)**		**OSB1, OSB2**		
***Fragaria x ananase* (Strawberry)**	**FaMYB1***			
***Petroselinum crispum* (Parsley)**				**CPRF1*, CPRF2, CPRF5-7 (all bZIP)**
***Pisum sativum* (Pea)**		**A, A2**		
Proanthocyanidin biosynthesis				
Arabidopsis thaliana	**TT2**	**TT8**	**TTG1**	**TTG2 (WRKY), TT1 (WIP zinc finger)**
Phlobaphene biosynthesis				
***Zea mays* (Maize)**	**P**			

In maize, all the genes encoding the core enzymes of the flavonoid pathway (Fig. 3.2) are regulated by a combination of the R2R3 MYB factors C1 or PL and the

HLH factors R or B.[8] The promoters of the *a1*[21,22] and *a2*[23] genes, encoding maize DFR and LDOX/AS, respectively, have been molecularly dissected and shown to be modular in structure, with binding sites for the R2R3 MYB factor C1 and containing the conserved ARE (Anthocyanin Regulatory Element) sequence. The *in vivo* effect of transposon insertions and footprints in the *a1* ARE underscore the significance of this *cis*-acting element for the regulation of flavonoid biosynthetic genes.[24] Although early studies suggested the presence of E-box-like elements recognized by R in the *bz1* promoter,[25] binding of R to DNA has not yet been possible despite significant efforts by the Chandler and Grotewold labs. This is consistent with the sequence divergence of the putative "basic" region associated with the HLH motif of R or B. Indeed, our current model suggests an indirect recruitment of R to the ARE, through as yet unidentified factors (Fig. 3.3A). The physical interaction between C1 and R/B involves the R3 MYB repeat of C1 and the N-terminal region of R.[26,27] Four solvent-exposed residues in the MYB domain of C1 are central to the specificity of the interaction between C1 and R. The transfer of these residues to the distantly related MYB P, which is an R-independent regulator of 3-deoxy flavonoid biosynthesis, is sufficient to expand the P-regulatory activity to include C1-regulated genes.[27] These findings suggest a model involving the gain and loss of protein-protein interactions by changes in a few residues for the evolution of novel branches of flavonoid biosynthesis.

In petunia, the MYB and HLH factors AN2 and JAF13 also cooperate to activate anthocyanin biosynthesis.[8] Different from maize, however, in petunia, snapdragon, *Arabidopsis*, and probably many other dicots, the flavonoid biosynthetic genes are not all coordinately regulated, and a clear distinction exists in expression between "early" and "late" genes.[8,9] Interestingly, the MYB and HLH factors are exchangeable between maize and petunia,[28,29] indicating a high level of conservation in the regulatory mechanisms by which distantly related plants control the flavonoid pathway. However, recent findings either suggest that there are differences in the way petunia and maize regulate the pathway, or that key players remain to be identified. From a sequence identity perspective, JAF13 is the evident ortholog of R, and as such, it interacts with AN2. However, Petunia also expresses another HLH factor, AN1, which is transcriptionally regulated by AN2 and JAF13 (Fig. 3.3B).[30] Both AN1 and JAF13 have the ability to activate DFR. In addition, AN1 plays an important role in the control of vacuolar pH and in seed coat cell morphology,[30] functions that have not yet been investigated in maize. Interestingly, *an1* is more related to the maize *intensifier1* (*in1*) gene than to *r* or *b*. *in1* was genetically identified as a possible inhibitor of the anthocyanin pathway, which intensifies aleurone pigmentation when mutated.[31] The complex splicing pattern reported for *in1*[31] has made it difficult to investigate molecularly how it participates in the

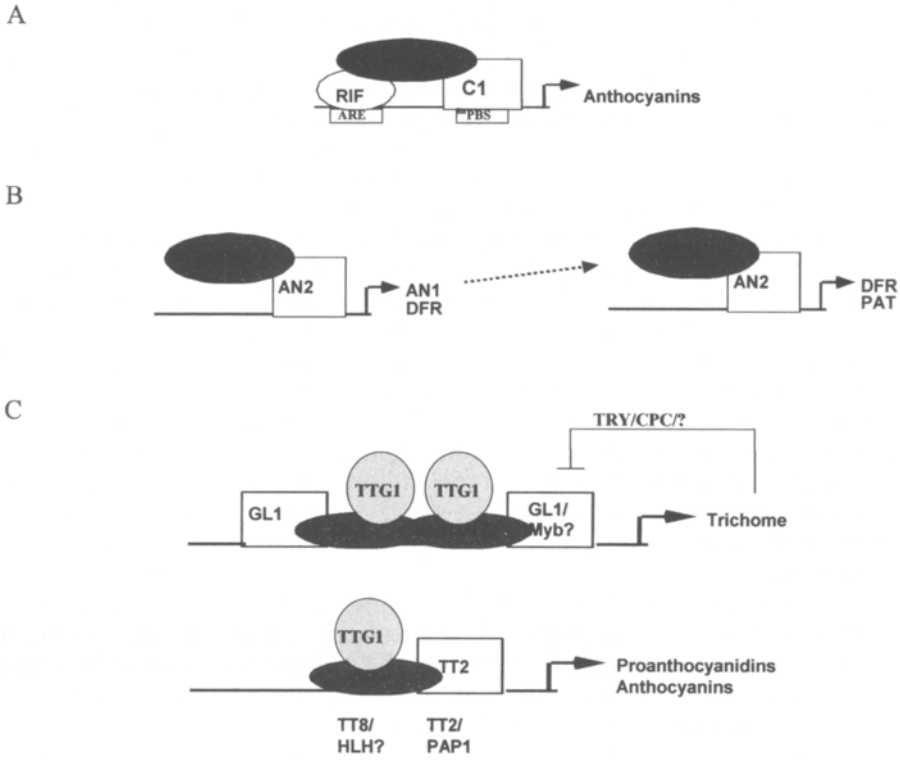


Figure 3.3: Current models for the transcriptional control of anthocyanin biosynthetic genes in maize (A), petunia (B), and *Arabidopsis* (C). The Petunia model is based on Spelt *et. al.*,[30] and the regulation of trichome formation was adapted from Payne *et. al.*[35]

[ha]PBS corresponds to the high affinity P binding site.[22]

regulation of anthocyanin accumulation, yet might be the reason for its function as an inhibitor.

In addition to the cooperation between MYB and HLH factors, the identification of the AN11 protein as containing WD40 repeats provided the first evidence of the involvement of factors with this domain in flavonoid regulation.[32] Mutations in *an11* result in no other visible phenotype than non-pigmented flowers, even though *an11* is expressed in all tissues. The cytosolic AN11 protein appears to act upstream of AN2 since the over-expression of AN2 in mutant *an11* petals activates a luciferase reporter driven by the *dfr* promoter.[32] The first identified regulatory mutant of the *Arabidopsis* flavonoid pathway corresponds to *ttg1*, which encodes a WD40 protein with a high sequence identity to AN11.[33] *ttg* mutants not only lack the typical pigmentation characteristic of *Arabidopsis* seed coats, provided by the accumulation of proanthocyanidins and tannins, but also lack trichomes (hence the name *transparent testa glabra*). *ttg1* mutants are also devoid of anthocyanins in the vegetative tissues and are deficient in the accumulation of seed mucilage. All the phenotypes of *ttg1* mutants can be complemented by the expression of the maize *R* gene.[34] These findings link in unexpected ways the formation of trichomes and the accumulation of flavonoid pigments. This relationship is further validated by the participation of distinct, yet related, MYB and HLH proteins in both processes. For the formation of trichomes, the R2R3 MYB protein GL1 interacts with the HLH protein GL3, probably with other HLHs, and also with R (Fig. 3.3C).[35] Indeed, the residues involved in the specificity of the interaction between C1 and R are highly conserved in GL1,[27] and GL1 comes close to C1 in phylogenetic reconstructions that include maize and *Arabidopsis* MYB sequences. However, the functions of GL1 and GL3 appear to be associated solely with the formation of trichomes, and not with the accumulation of flavonoid pigments. It is the combinatorial action of the TT2, an R2R3 MYB, and the HLH protein TT8, which results in the accumulation of proanthocyanidins in *Arabidopsis* seed coats (Fig. 3.3C).[36,37] While a physical interaction between TT2 and TT8 has not yet been demonstrated, the high respective identity with C1 and R suggests that these two proteins act in concert. Yet the ectopic expression of TT2 in roots induces the expression of TT8, a finding that strongly resembles the activation of *an1* by AN2.[30] TT2 is only expressed in the seed coat, and activation tagging recently identified another MYB factor, PAP1, capable of inducing pigmentation in all green tissues.[38] A partner for PAP1 has not yet been identified, although this MYB is also capable of interacting with the maize HLH, R.[39]

All these findings suggest a conserved network of protein-protein interactions resulting in the facilitation of MYB transcription factors to activate transcription of the corresponding target genes (Fig. 3.3). It is not clear, however, what the participation of the WD40 proteins might be. It was suggested[33] that TTG1 may contribute to the nuclear localization of the MYB and HLH factors. If so, this is

unlikely to happen through the HLH factors, since nuclear localization signals have been identified in R.[40] Strikingly, it is the HLH partner in the GL3-GL1 complex that physically interacts with TTG1.[35] Clearly, more studies are needed to further establish the mechanisms by which the cooperation of MYB/HLH/WD40 proteins result in the control of anthocyanin biosynthesis.

REGULATING THE REGULATORS

A variety of factors including light, hormones, and stress can induce the accumulation of anthocyanins.[2,41] How are the known regulators controlled by these environmental stimuli, and/or by developmental signals that result in fascinating temporal and spatial patterns of pigment accumulation? Recent reviews have failed to link the major plant signaling pathways to what is known on the regulation of anthocyanin accumulation, an ubiquitous response to many of the signals and stress conditions that plants encounter.[42]

Light is essential for plant development and survival. However, too much light, the wrong type of light, or light at the wrong time can cause photo-oxidative damage. The production of anthocyanins is one of the multiple strategies that higher plants employ to diminish the damaging effects of high photon flux.[43] Consistent with this, many of the *Arabidopsis* phenylpropanoid biosynthetic genes are under circadian regulation, peaking just before dawn.[44] In these circadian studies, PAP1 (Fig. 3.3) shows a similar pattern of expression as the phenylpropanoid genes, suggesting that under these conditions, it might be a master regulator of the anthocyanin genes.[44]

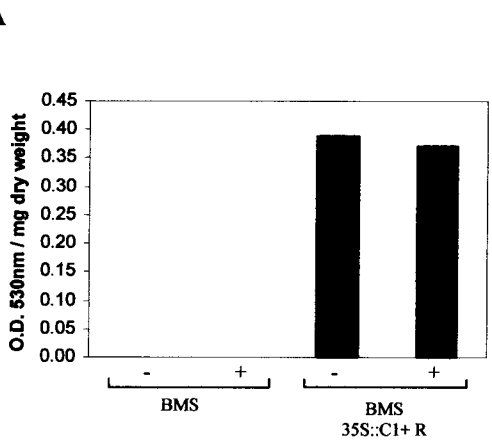
In *Arabidopsis*, light affects the expression of about a quarter of all genes. Not surprisingly, several phenylpropanoid and anthocyanin biosynthetic genes were among those induced by the light treatment of *Arabidopsis* seedlings.[45] Phytochromes are known to mediate signaling responses by directly activating transcription factors, deactivating nuclear repressors, and modifying cytosolic proteins.[46] Five phytochromes (PhyA-E) have been identified to date in *Arabidopsis*, and the loss of responsiveness in *phyA* and *phyA/phyB* double mutants supports a PhyA signaling mediated control of anthocyanins. Indeed, several flavonoid and phenylpropanoid biosynthetic genes are among those induced by far-red light, and dependent on PhyA.[47] The presence of a PhyA-regulated transcriptional network[47] with the stability of some of the participating transcription factors regulated by the COP9 signalosome[48] is well established. However, the specific mechanisms by which light, PhyA, and the COP9 signalosome modulate the activity or stability of the known anthocyanin regulators are yet to be elucidated.

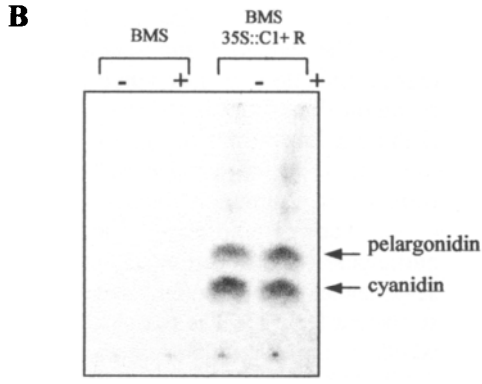
Recent studies suggest that in maize, the MYB regulators C1 and PL are central to the response of maize aleurone and mesocotyl, respectively, to light. Indeed, most of the detected induction of the CHS (*c2*) and DFR/A1 (*a1*) transcripts could be attributed to the increased transcription of these MYB regulators.[49] This is

similar to what has been observed in other plant species, such as parsley and *Arabidopsis*.[50,51] A prediction of these models suggests that the effect of light on anthocyanin accumulation is solely mediated by increased transcription of the MYB regulators. Thus, cells expressing the C1 and R from a light-insensitive *CaMV 35S* promoter should not be affected by light. Interestingly, maize Black Mexican Sweet (BMS) cell cultures or calli expressing 35S::C1 and 35S::R,[12] treated with strong white light, display a much darker red pigmentation than observed in cells grown in complete darkness. No significant increase in the steady-state mRNA levels for *a1* or *c2* were observed in light- versus dark-grown cell cultures; neither was a qualitative or quantitative alteration in the accumulated anthocyanins observed (Fig. 3.4). Nevertheless, *in vivo* reflectance measurements showed a significant shift in the hue of the pigments accumulated, with an unexpected "yellowing" (evidence of which is seen in the decrease in the +a value and increase in the +b colorimetric CIELAB value, Fig. 3.4) of the pigments in the light. Possibly related to these findings, blue light has minor effects on *c1* or *a1* expression in maize aleurones, yet blue light induces substantial pigmentation.[49] These findings suggest that the pigment properties of anthocyanins can be controlled independently of the expression of the known regulators. How this control is achieved and what it may involve in molecular terms remains to be established.

Plant hormones can also influence anthocyanin accumulation.[52] There is a reasonably good understanding on the regulation of *c1*, whose expression is induced in the aleurone during maize seed development, by the action of the *viviparous1* (*vp1*) gene, abscisic acid, and light.[53,54] The promoter of the *c1* gene has *cis*-elements responsible for ABA/VP1 regulation,[55] as well as independently functioning elements for regulation by light. VP1 is a transcription factor of the B3 domain family, which binds to the RY element present in the *c1* promoter and in several other seed-specific genes. The specific mechanisms by which ABA and VP1 cooperate to activate *c1* expression are still poorly understood. The *Arabidopsis* ABI3 protein is similar to VP1, and *abi3* mutations are complemented by VP1,[56] yet it is not known whether ABI3 regulates any of the *Arabidopsis* flavonoid regulatory genes.

Post-transcriptional regulation of the regulators has also been demonstrated. *lc,* one member of the *r/b* gene family, has upstream-untranslated open reading frames (uORF) that negatively regulate its translation.[57] In addition, the 5' UTR of the *lc* mRNA has the ability to form a secondary structure that further represses *lc* translation.[58] Similarly, transcripts of *sn*, another member of the complex *r/b* gene family of HLH regulatory proteins, are regulated by alternative splicing of the 5' UTR.[59] It is intriguing why these genes are subjected to the precise regulation that these studies suggest, particularly given that the appropriate alleles of the *b* gene, which encodes proteins functionally equivalent to R,[60] have the ability to control pigmentation in almost all maize cell types, yet do not seem to be subjected to similar levels of regulation.





C

	Light	L		a		b		h_{ab}	C*
		avg.	s.d.	avg.	s.d.	avg.	s.d.		
BMS::35SC1+R	-	21.95	± 0.58	8.26	± 0.23	-0.1	± 0.19	359°	8.26
	+	15.85	± 0.76	5.2	± 0.23	1.57	± 0.1	17°	5.43

Figure 3.4: Light induces color changes, but does not affect the type or quantity of anthocyanins in maize BMS cells expressing the R & C1 regulators from the *CaMV 35S* promoter.[12] **A.** Absorbance at 530 nm of methanol - HCl extracts of lyophilized material. **B.** TLC of acid-hydrolized methanol extracts extracted with isoamyl alcohol. Cellulose TLC plates were run in water:formic acid:HCl (10:30:3). **C.** Color differences of the red maize BMS cells R & C1[12] in the dark (-) and light (+) measured by colorimetry. Hue angles (h_{ab}) were calculated as according the CIELAB color space system, plotting +/-a and +/-b values on a 4 quadrant plot (+a = red; -a = green; +b = yellow; -b = blue; L: lightness is maximum at 100). C* is the metric chroma, the saturation value and is calculated by the formula $(a^2+b^2)^{1/2}$.[70]

BEYOND MODEL SYSTEMS

The temporal and spatial diversity of pigmentation patterns displayed by leaves and flowers has surely not escaped anybody's attention. Yet, despite flavonoid biosynthesis being one of the best described pathways in plants, almost nothing is known regarding how plants manage to use anthocyanin in such a variety of ways. The following examples provide exciting opportunities to link what has been learned from model plant systems and the physiology of anthocyanins in nature.

The red coloration of leaves in the autumn has recently been suggested to participate in protection against photo-oxidative damage prior to chloroplast senescence, giving enough time for nutrient retrieval, particularly nitrogen, from the senescing leaves.[61] It is not clear what triggers these spectacular color changes, and whether similar regulatory networks as described above are affected by shortening day length, or by changing light intensities. A similar photo-protective function of anthocyanins has been proposed for the leaves from some plants that accumulate anthocyanins constitutively, such as, for example, the leaf undersurface of many tropical herbs with extreme shade habitats.[62] It is possible that lower photosynthetic activity or early stages of chlorophyll catabolism participate in the induction of anthocyanins.

Another fascinating example of the complex regulation of anthocyanins is provided by the phenomena of color change in fully developed flowers. Floral color change is widespread in the flowering plants,[63] and appears to provide unique color cues for the interaction of plants with pollinators. For example, before the color change, flowers might have receptive stigmas offering pollen and nectar reward to pollinators, while after the change, flowers might be sexually non viable. Yet, in some cases, the retention of the post-change flowers may serve as long-distance attraction to pollinators. While ethylene has been proposed to be sometimes involved in the *de novo* synthesis of anthocyanins that is frequently associated with color phase change, this is not the case in *Viola cornuta* cv. Yesterday, Today, and Tomorrow flowers, which change from white to purple over 5-8 days because of a dramatic increase in malvidin. In this plant, the color change is dependent on light and on the presence of pollen on the stigma.[64] Interestingly, white flowers accumulate significant levels of flavonols, suggesting that the block in the pathway is not in the early enzymes (Fig. 3.2). Indeed, the juvenile to mature phase transition in English ivy (*Hedera helix* L.), associated with the loss of anthocyanin pigmentation in stems and petioles, appears to be due to a block in DFR in mature phase tissue.[65] It will be interesting to establish whether the convergent evolution of color phase change[63] is the result of the same step in the pathway being affected, or to multiple mechanisms leading to the same phenotypic effect.

Tulip breaking, the oldest example of a plant virus disease, caused by the TBV potyvirus, provides one of the best examples of how a pathogen, can interfere with the accumulation of anthocyanins, inducing the legendary "tulipmania" in

humans.[66] The infected flowers display spectacular pigmentation patterns unrivaled by the heritable color designs selected by breeding, and presumably determined by unknown developmental cues. At what level color breaking interferes with the accumulation of anthocyanins is unknown. Perhaps, the pigmentation patterns are a consequence of the ability of viruses to silence host gene expression,[67] which in this case could be targeting biosynthetic enzymes or regulatory factors of flavonoid biosynthesis.

Color perception can also be influenced by the physical properties of the cells where anthocyanins accumulate. An example is provided by the effect of the *Mixta* gene in snapdragon, which controls conical cell shape. Petal cells lacking *Mixta* function are palely pigmented because of the way light reflects, which could have significant consequences in the ways pollinators are attracted.[68]

These examples show that despite our extensive knowledge on flavonoid biosynthesis and regulation, we still know little on how development or abiotic/biotic stresses affect pigmentation.

SUMMARY

As we begin to unravel the molecular mechanisms of the regulation of anthocyanin biosynthesis, we must remember that evolution and selection had significant opportunities to explore new ways to color plants, flowers, and seeds. Model plant systems such as maize, petunia, and *Arabidopsis* will continue to provide the framework to understand how pigment accumulation is controlled. However, it is important to investigate how nature has exploited variations in these central prototypes to provide the amazing diversity found today in the type and distribution of anthocyanin pigments.

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Chapter Four

LOCALIZATION OF PLANT MYROSINASES AND GLUCOSINOLATES

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INTRODUCTION

Glucosinolates constitute a group of sulfur-containing secondary metabolites characteristic of 15 families of the order Capparales, with the highest diversity in the family Brassicaceae.[1] These compounds consist of a thioglucoside moiety linked to a variety of amino acid-derived side chains.[2-4] The enzyme myrosinase (ß-thioglucoside glucohydrolase, E.C. 3.2.3.1) catalyzes cleavage of glucosinolates to aglucones that decompose to form more or less toxic products, such as isothiocyanates (mustard oils), thiocyanates, nitriles, or epithionitriles.[5] Myrosinase activity has been detected in all glucosinolate-containing taxa that have been investigated. While glucosinolates are sometimes regarded as storage substances or precursors involved in intermediary metabolism, the myrosinase-glucosinolate system is important for plant-insect interactions.[6] The localization of myrosinase and glucosinolates has been debated for a long time and is of interest because it provides a way to approach the functions of the myrosinase-glucosinolate system. Since many defense compounds are toxic to the plant itself, plants must be able to cope with these substances without poisoning themselves. One strategy is to store toxins as inactive precursors separately from the activating/degrading enzymes. Glucosinolates and myrosinase have to be brought together in order to interact because they are located in different cells, with myrosinase mainly located in special idioblasts called myrosin cells. The activation of this "mustard-oil bomb" follows tissue disruption caused by, for example, insect herbivory.

Arabidopsis thaliana (L.) Heynh. is today the most useful model system in plant research, and in order to be able to generalize results from this species, it is important to investigate its special characteristics, such as the myrosinase-glucosinolate system. In this chapter, we present the localization of the myrosinase-glucosinolate system in *Arabidopsis* and other plants and discuss *in vivo* degradation and transport of glucosinolates.

LOCALIZATION OF GLUCOSINOLATES

The first isolation of a glucosinolate, sinapisine (later known as sinalbin) from *Sinapis alba* seeds, was reported in 1831.[7] Attempts to localize glucosinolates at the tissue level have been difficult due to the water solubility of the compounds. However, all studies have indicated a vacuolar localization of glucosinolates. Grob and Matile[8] isolated vacuoles by density gradient centrifugation from plasmolyzed root tissue of horseradish (*Armoracia rusticana* Gaertn. Mey. Sherb., syn. *A. lapathifolia* Gilib.), and found that the glucosinolates were located there. This study assumed that all phenolics in the tissue were located in the vacuoles as well. Helmlinger et al. provided evidence for the vacuolar localization of glucosinolates,

newly synthesized from [^{14}C]-L-tryptophan, by isolating vacuole-rich fractions of horseradish and using acid phosphatase as a marker.[9]

Wei et al., using x-ray microanalysis on cryosections of roots of *S. alba*, found high concentrations of sulfur, indicating the presence of glucosinolates in the vacuoles of both cortex cells and root cap cells.[10] The distribution of glucosinolates paralleled the differentiation of the root tip tissues, as only cells that were more than 0.2 mm from the root tip had well-developed vacuoles and contained glucosinolates. Yiu et al. used N,2,6 trichloro-*p*-benzoquinoneimine to detect glucosinolates in methanol extracts on silica gel plates.[11] These authors also carried out histochemical studies of hand-cut sections from *Brassica napus* cotyledons. The detected color reaction was confined to protein bodies (vacuoles) in virtually all cells. In immature fruits of *Carica papaya,* nearly 10% of the dry latex was found to be benzylglucosinolate.[12] The only glucosinolate that has been localized immunohistochemically is the main aliphatic glucosinolate sinigrin of *Brassica juncea* embryos from imbibed seeds.[13] Sinigrin was present in vacuoles of aleurone-like cells, i.e., in most cells of the embryo, but not in myrosin cells. In contrast, Thangstad et al. found accumulation of radioactivity after feeding of immature siliques with ^{3}H-labelled glucosinolate precursor in putative myrosin cells in the developing embryo.[14] However, it was not fully clarified whether the signal was due to glucosinolates or to precursors/degradation products at that developmental stage. Other evidence that glucosinolates are not present in myrosin cells comes from the fact that specific Rnase ablation of myrosin cells in seeds of *B. napus* does not change the glucosinolate pattern.[15]

In *Arabidopsis* flower stalks, glucosinolates are highly enriched in groups of certain sulfur-rich "S-cells", located externally to the vascular bundles.[16] The sulfur was identified by energy dispersive x-ray microanalysis with a scanning electron microscope. Direct freezing in liquid nitrogen before freeze-drying and sectioning circumvented the problem of water-solubility. The glucosinolate content of these cells was confirmed by enzymatic hydrolysis by myrosinase of sap collected from the S-cells by a glass microcapillary, and the concentration of glucosinolates was found to be above 200 mM.[16] This combination of x-ray microanalysis and cell sap analysis is probably the most reliable method available to date for determining glucosinolate localization. In addition to the high sulfur-content in the S-cells, a relatively high sulfur-content was also identified in the epidermis of the flower stalk. The S-cells are giant cells located between the phloem and the endodermis. The endodermis is also called the starch sheath (Fig. 4.1A,B). In the young flower stalk, they are probably the longest cells (up to 1 mm), and they are wide and thin-walled.[16] A group of cells outside the phloem in *Arabidopsis* stems has been referred to as "phloem-cap cells",[17] and these include the S-cells. We have not been able to detect any obvious anatomical equivalent to these cells in leaves. By transmission electron

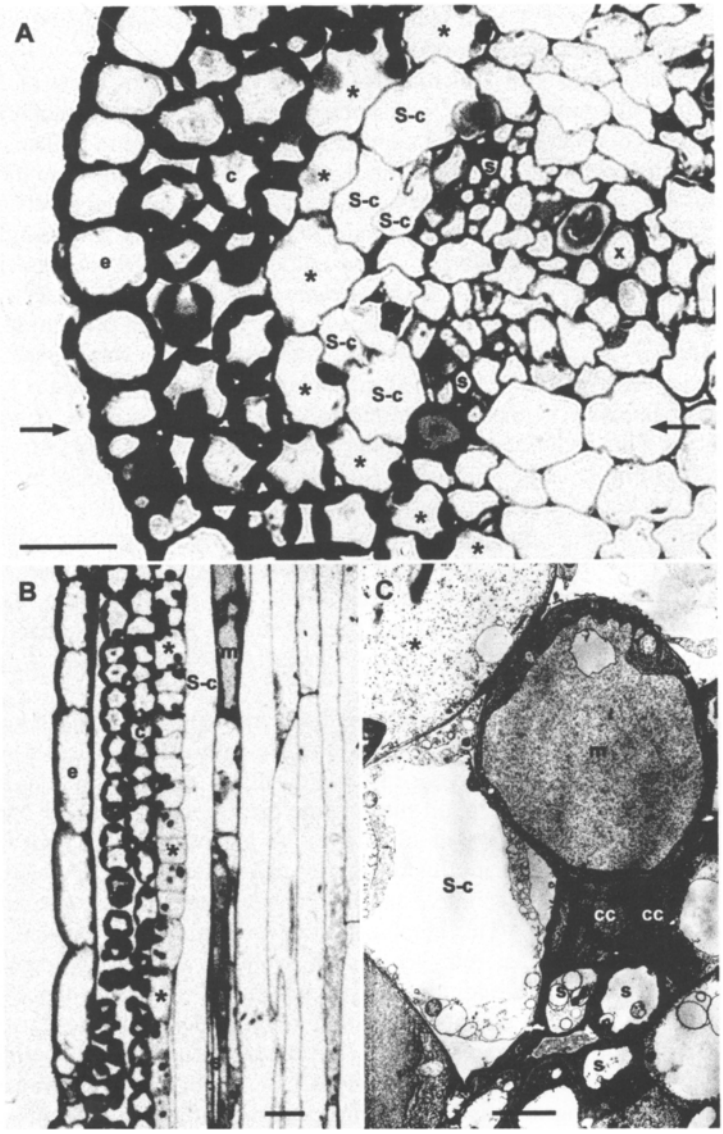
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Figure 4.1: The mustard oil bomb in flower stalks of *Arabidopsis thaliana* consists of S-cells (with glucosinolates) and adjacent myrosin cells (with myrosinase). This is illustrated by transverse (A,C) and longitudinal (B) sections of a pedicel, analyzed by light microscopy (A,B) and transmission electron microscopy (C). The myrosin cells (m) are in contact with the S-cells (S-c), situated inside the starch sheath (*) (A,B,C). The myrosin cells are located peripherally in the phloem tissue; other cells of the phloem include sieve elements (s) and companion cells (cc, in (C) only). The starch sheath is the innermost layer of the cortex (c), of which the outer cells have chloroplasts, in contrast to the surface layer, epidermis (e). In (A) the xylem (x) forms the vascular tissues together with the phloem. The longitudinal section in (B) is oriented along the line between the arrows indicated in (A). For methods, see.[18] Size bars equal 25 μm in (A,B) and 2.5 μm in (C).

microscopy studies, it has been found that the S-cells have huge central vacuoles surrounded by a very thin layer of cytoplasm with few organelles.[18] The S-cells appear to degenerate relatively early compared to the surrounding cells, and in some S-cells, several vesicles of different sizes are present in the cytoplasmic layer (Fig. 4.1C). These vesicles could be involved in trafficking of glucosinolates, in analogy to what has been observed for other substances.[19] The S-cells are often situated in direct contact with myrosin cells in the peripheral part of the phloem (Fig. 4.1A-C; see below).[18]

Recent articles describing glucosinolate biosynthetic genes in *Arabidopsis* have included promoter-GUS fusions that indicate a vascular localization of corresponding transcripts.[20,21] However, in these studies, no detailed histological analyses were carried out. It has been reported that promoter-GUS fusion analyses may give overrepresentation of genes expressed in the vascular tissue,[22] so determination of tissue specificity based on this technique alone should be regarded with some precaution. This is also because a transgenic approach always involves a risk of missing regulatory elements upstream or downstream of the cloned DNA.

THE MYROSIN CELL

History and Structure

"Myrosyne" was first characterized from *Brassica nigra* seeds in 1839 by Bussy as a substance analogous to albumine and emulsine, i.e., a protein, necessary for the release of essential oil from mustard seed.[23,24] The word myrosyne stems

from Greek muron (μυρον), volatile oil, and syn (συν), with. This name was derived from Bussy's conclusion that in order to produce the oil, myrosyne had to be together with "l'acide myronique", which he isolated as "myronate de potasse" (sinigrin) from the seeds.

In Brassicaceae species, Heinricher identified in 1884 a special protein-rich cell type differing in morphology from adjacent cells and showing high light refraction and affinity to different histological stains and protein-reagents.[25] The special refraction was observed after boiling in water and treatment with alcohol. The term myrosin cell was first suggested by Guignard,[26] and has been used for this special type of cell that stains strongly with protein dyes such as Millon's reagent[27] and Aniline Blue Black[28] (Fig. 4.2A). The myrosin cell is a special type of idioblast, a term that was first used by Sachs for mostly single cells, deviating by shape or content from the surrounding homogeneous cells in a tissue.[29] It is generally believed that idioblasts have evolved to have different functions in different plant taxa,[30] and in Capparales the characteristic idioblast type is the myrosin cell.

Myrosin cells are scattered in seeds, roots, stems, leaves, and flowers.[26] They have been found in all the main plant tissue types: ground tissue, vascular tissues, and epidermis.[18,31] The myrosin cells in the phloem of the vascular tissues are usually elongated, and in the ground tissue they may have the same shape as the surrounding cells or may be long and slender due to intrusive growth.[25,32,33] Sometimes they occur in small groups or in pairs.[18,32] Myrosin cells are easier to identify in younger vegetative tissues than in older because the ER becomes less prominent in the cytoplasm and the protein content in the vacuoles diminishes.[33-35] In the Brassicaceae, genera have been classified according to the occurrence of myrosin cells in the ground tissue, the vascular tissue, or both.[36] Jørgensen used occurrence of myrosin cells as a criterion for including families in the order Capparales.[31] Subsequently, the occurrence of myrosin cells in fourteen glucosinolate-containing families of Capparales was summarized: all except one had idioblastic myrosin cells and/or stomatal myrosin cells, and three (Bataceae, Resedaceae, and Caricaceae) had stomatal myrosin cells exclusively.[35] In Brassicaceae, Schweidler found stomatal myrosin cells in a few species of *Arabis* but not in *Arabidopsis thaliana* (L.) Heynh. (syn. *Arabis thaliana* L. and *Stenophragma thalianum* Celak.), which had myrosin cells in the phloem.[37] Our later investigations of *Arabidopsis* have not identified stomatal myrosin cells (Andréasson E and Jørgensen LB, unpublished). The ultrastructural study of the development of stomata in *Arabidopsis* did not reveal the myrosin cell character of these stomata, even though loose electron-dense material in the vacuoles of a few guard cells could be identified in the micrographs.[38]

Ultrastructurally, the vacuolar content of all myrosin cells is electron dense. In mature embryos, myrosin cells can be distinguished from the surrounding cells by the absence of globoids (areas containing inorganic salts) in the protein bodies, which are vacuoles (Fig. 4.2B).[33,39-41] During embryogenesis, the vacuoles of myrosin cells are filled with protein earlier than the vacuoles of the surrounding cells.[39, 42] This also applies to the stomatal myrosin cells in embryos of *Carica* spp.[35] In myrosin cells in vegetative tissues derived from the stem apical meristem, i.e., young stems and leaves, the vacuolar content is finely granular and moderately electron-dense. The cytoplasm is characterized by extensive, distended rough endoplasmic reticulum (rER) with content similar to the vacuoles (Fig. 4.2C).[18,31,32] These ultrastructural characteristics, together with the vacuolar affinity for protein stains in light microscopy, define the myrosin cell structurally. For mature embryos, the rER-character is not applicable due to the structure of the cytoplasm where oil-bodies are an almost exclusive component, and organelles and membranes are vestigial (Fig. 4.2B).

During seed development in *B. napus*, the first myrosinase-containing cells appear approximately 20 days after pollination. The number of these cells gradually increases until a level of 2-5% of the total number of embryo cells is reached.[43] In green expanded cotyledons, approximately 0.1 % of the cells are myrosinase-containing, and the corresponding number in leaves is 0.05 %.[18] A large proportion of the myrosinase-containing cells are located peripherally in the ground tissue of the axis and cotyledons (Fig. 4.3A).[18,43,44]

Myrosin Cells and Myrosinase Expression

Staining of consecutive sections with histochemical reagents for protein and antibodies against myrosinase showed that myrosin cells actually contain myrosinase (Fig. 4.2D,E).[18,43,44] By electron microscopy immunogold labelling studies, the enzyme was localized to the interior of the vacuoles of the myrosin cells (Fig. 4.2F).[18,40,41] It has also been shown by several *in situ* hybridization experiments that myrosinase transcripts are located in these cells (Fig. 4.2G),[6] demonstrating that the cellular localization of myrosinase is due to transcriptional regulation and not to a transport process. No evidence for transport of myrosinase currently exists. These methods for detection and identification of myrosin cells and myrosinase expression are illustrated (Fig. 4.2). For a historical overview of myrosinase localization studies, see Bones and Rossiter.[45]

Transmission electron microscopy analyses of ultrathin sections have indicated that there are several vacuoles in myrosin cells of embryos. These vacuoles contain myrosinase, as revealed by immunogold labelling studies, and have been referred to as myrosin grains.[40,41] Recently, confocal laser scanning immuno-microscopy analysis with the anti-myrosinase monoclonal antibody 3D7 has been

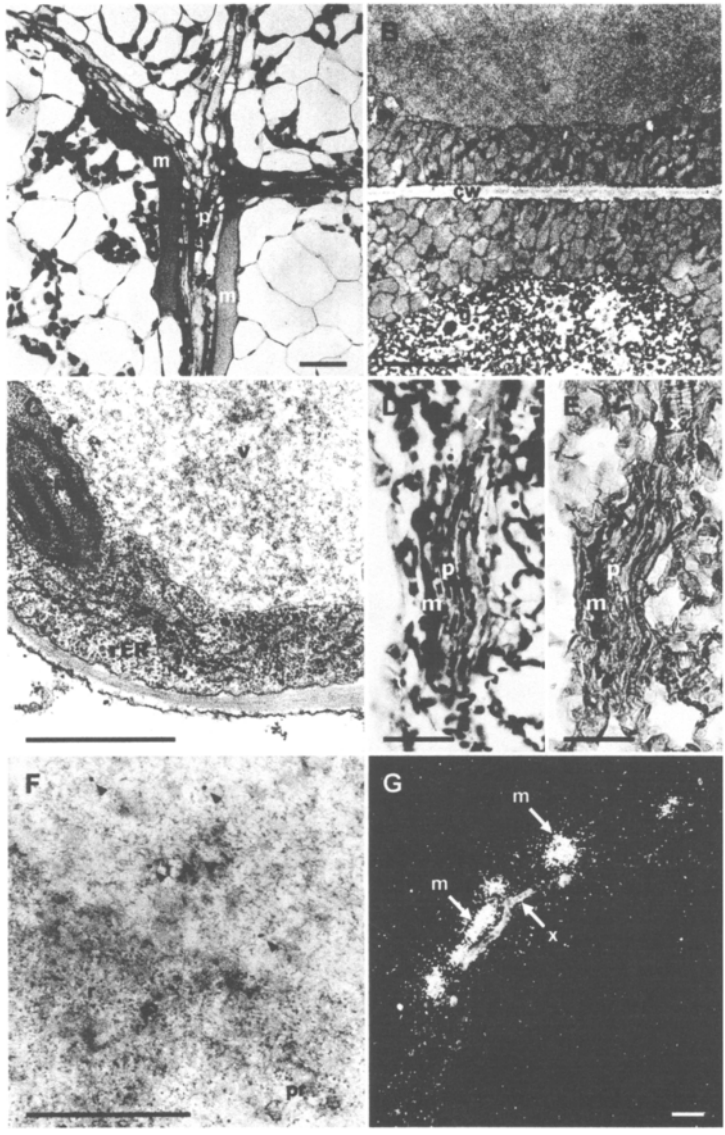
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Fig. 4.2: Identification and localization of myrosin cells and myrosinase expression by histochemical staining in light microscopy (LM), by transmission electron microscopy (TEM), by immunolocalization, and by *in situ* hybridization, in *Arabidopsis thaliana* (A,C-G) and *Sinapis alba* (B). (A) LM analysis of Aniline Blue Black histochemical staining of protein in plastic-embedded leaf highlights the two myrosin cells (m) in the phloem (p) of a vascular bundle, also including xylem (x). (B) TEM-analysis of a cotyledon from an imbibed seed shows the myrosin cell (m, above) with homogeneous protein in the vacuole (v). In the non-myrosin cell, below the common cell wall (cw), the vacuole (v) contains globoids (g). In both cells, the cytoplasm is dominated by oil-bodies (ob). (C) TEM-analysis of a myrosin cell from a leaf with moderately electron-dense, homogeneous material in the vacuole (v) and distended rough endoplasmic reticulum (rER) in the cytoplasm. Also a chloroplast is present (cp). (D) Histochemical staining, as in (A), of paraffin-embedded leaf identifies a myrosin cell (m) peripherally in the phloem (p). (E) Immunohistochemical staining of a consecutive section to (D) with the anti-myrosinase antibody 3D7 shows the presence of myrosinase in the myrosin cell identified in (D). Both (D) and (E) also show the xylem (x) in the vascular bundle. (F) TEM immunocytochemical identification of myrosinase with the 3D7 antibody by immuno-gold labeling (arrowheads) in the vacuole (v, upper right half of the picture) of a myrosin cell. In the cytoplasm (lower left half), ribosomes (r) and polysomes (pr) are discernible. (G) *In situ* hybridization with a *TGG2* myrosinase probe visualizing transcripts in myrosin cells (m) in the phloem of a leaf. The xylem (x) appears bright in dark-field microscopy. For experimental details, see,[18] except for (G), see.[48] Size bars equal 25 μm in (A,D,E,G) and 1μm in (B,C,F).

used to study the spatial organization of the myrosin grains in myrosin cells of the mature *B. napus* embryo.[18] This analysis revealed that the myrosin grains within one cell actually seem to constitute one continuous reticular vacuole, a "myrosin body". One study with transmission electron microscopy showed that an undefined area in the vacuole of non-myrosin cells of *B. juncea* mature embryo cross-reacted with another anti-myrosinase antibody,[13] but this is probably not a myrosinase-specific signal.[46]

In addition to myrosinase expression in myrosin cells of ground tissue and phloem, there are reports of myrosinase transcript and protein in *B. napus* guard cells of germinating embryos and young leaves. This was discovered by use of the anti-myrosinase antibody 3D7 and non-radioactive *in situ* hybridization using a myrosinase B specific probe.[18,43] No myrosinase expression in guard cells has been reported in older *B. napus* tissues. A recent report used an *Arabidopsis* myrosinase (*TGG1*)-promoter GUS construct to show that, in addition to the myrosin cell expression in the phloem, a myrosinase expression in guard cells is present.[47] Because this reporter gene is highly stable, it is possible that there is a transient myrosinase expression in guard cells in *Arabidopsis*, similar to that in *B. napus*. This is supported by *in situ* hybridization with a *TGG1* probe and immunolocalization with anti-myrosinase antibodies 3D7 and K505 in *Arabidopsis*, showing only myrosinase expression in phloem myrosin cells.[18, 47, 48] Also, because the myrosinase activity and western signal with 3D7 correlate in *Arabidopsis*,[18,49] it seems reasonable to believe that the majority of myrosinase is located in phloem myrosin cells.

There are other examples showing that the presence of structurally defined myrosin cells is not a prerequisite for myrosinase activity. For instance, Bones and Iversen found myrosinase activity in roots of 48h germinating seedlings of several Brassicaceae species, at which stage myrosin cells could no longer be distinguished.[33] In *Carica papaya*, Tang found myrosinase activity in the gelatinous outer layers of the seed coat,[12] but no myrosin cells have been detected in this tissue.[35] Myrosinase activity is also present in some fungi, bacteria, and insects (see references in;[45] see also chapter 6 in this volume by Rossiter).

Definition of the Myrosin Cell

We use the term myrosin cell structurally (see above), and not functionally (i.e., if a cell contains myrosinase it should be called a myrosin cell). This structural definition should be used because "myrosin cell" is a well-established anatomical term for this special idioblast. We do not find it useful to coin a specific term for a cell just because a certain protein is expressed in it, especially now in the post-genomic era, where possibly thousands of different proteins can be found in every cell. Husebye et al. named guard cells in *Arabidopsis* "myrosin guard cells" because myrosinase was found in these cells.[47] This is problematic since these cells do not

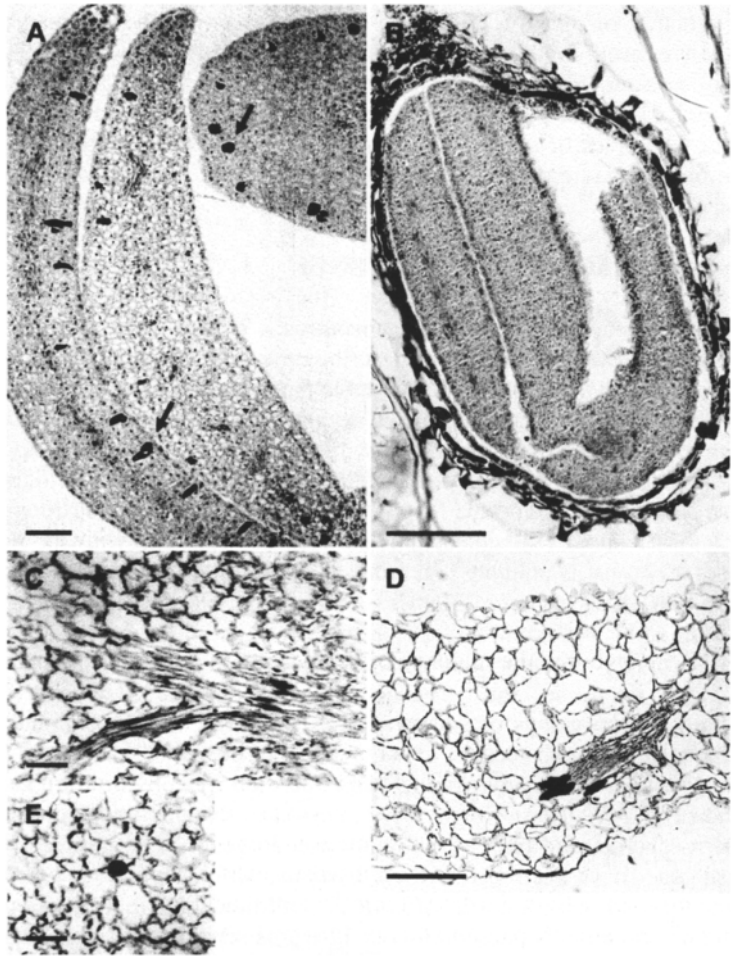

Fig. 4.3: Immunohistochemical analysis in *Brassica napus* (A,C,E) and *Arabidopsis thaliana* (B,D) of myrosinase by use of the antibody 3D7. Myrosinase is present in the ground tissue of cotyledons (left) and radicle (upper right) of *B. napus* embryo (A), while in *A. thaliana* seed no expression is detected (B); some cells in the seed coat retain unoxidized substrate. Arrows in (A) show examples of myrosinase-containing myrosin cells. In *A. thaliana* leaves, myrosinase is located in phloem myrosin cells (D), whereas in *B. napus* leaves myrosinase is in myrosin cells in the phloem (C), as well as in the ground tissue (E). For experimental details, see[18]. The size bars equal 100 μm.

have the structure of myrosin cells, like the structurally defined stomatal myrosin cells that have been found in many other Capparalean families and used as a taxonomic character. Furthermore, we do not expect plant biologists in general to adopt the term "myrosin guard cells" for structurally normal guard cells. Currently, there are no examples of plants having myrosin cells without myrosinase activity, and in the phloem and the ground tissue, the myrosin cells are the only myrosinase expressing cells.[18, 43, 44]

MYROSINASE ISOFORMS AND GENES

Myrosinase activity in plants is dependent on species, cultivar, age, and plant organ examined. Several reports have described the isolation and physical-chemical characterization of myrosinase.[6,45] It is notable that part of the myrosinase activity is insoluble after imidazole extraction of *B. napus* leaves, particularly after induction with methyl jasmonate,[50] and this is overlooked in most studies. Purified myrosinases have low specificity for different glucosinolates[51,52] and likewise, the three-dimensional structural data of *S. alba* myrosinase suggest low substrate specificity.[53] The number of glucosinolates does not correlate with the number of myrosinases.[6] Thus, it is unlikely that a development of substrate specificity has led to the evolution of myrosinases. Rather, the amount or accessibility of myrosinase activity seems to be important, as for example Ratzka et al. suggest that in diamond back moth herbivory, a competition between myrosinase and glucosinolate sulfatase activity may determine the efficacy of glucosinolate defenses.[54] Several electrophoretic examinations of isoforms of myrosinase have demonstrated distinct patterns that also vary with species, organ, and age of the plant.[6]

In *A. thaliana,* six myrosinase (thioglucoside glucohydrolase) genes have been found (*TGG1-6*). Two of them are expressed in the aboveground organs only, two are root-specific, and two are probably pseudo-genes.[48,55,56] Appreciably more myrosinase genes have been reported for *B. napus*, which contains at least 20 genes divided into three subfamilies: *MA, MB, MC*.[6] In all functional myrosinase genes, the exon/intron organization is conserved with 12 exons separated by 11 introns, except for the recently discovered root-specific myrosinases that have 13 exons.[6,55] Interestingly, three O-β-glucosidase genes and the myrosinase genes share the same overall exon/intron organization, although certain exons appear to be fused in certain genes.[6] The two root-specific myrosinases in *Arabidopsis* are actually the ones that are most similar to other β-glucosidases.[55] An unusual feature of all characterized myrosinase genes is the presence of a GC donor splice site instead of the common GT site.[55,57] Expression of *TGG1* transcripts seems to be COI1 dependent,[58] and expression of *MB* transcripts is down-regulated by salicylic acid.[59] This indicates a dynamic expression of myrosinase in relation to biotic stress. In extracts from *B. napus* seeds and seedlings, myrosinases in the subfamilies *MB* and *MC* are found in

complexes together with myrosinase-binding proteins (MBP).[60-63] The levels of *MBP* transcripts are, like certain glucosinolates, induced in response to wounding and jasmonate treatment.[50,64,65] The binding of MBP to myrosinase is the major factor for complex binding in extracts of *B. napus* seeds,[63] and might explain the decrease in soluble myrosinase activity found after jasmonate treatment.[50]

THE MUSTARD OIL BOMB

Grob and Matile found that glucosinolates were located in vacuoles.[8,66] They also found 30 % of the myrosinase activity in the vacuolar fraction and that myrosinase had a tendency to adhere to membranes. This prompted Matile[67] to present "die Senfölbombe", the mustard oil bomb, later modified by Lüthy and Matile.[68] The model proposed cells with glucosinolates that are localized in the vacuole and the apparently membrane-associated myrosinase in the cytosol, with the two components being separated by the vacuolar membrane. By tissue disruption, the substrate and the enzyme come into contact and thereby release the toxic product. This model has been modified[6,45] because myrosinase is localized in vacuoles in myrosin cells different from glucosinolate-containing cells. Investigations of flower stalks in *Arabidopsis* (Fig. 4.1) show that the mustard oil bomb exists, but its components are in adjacent cells – a two-cell bomb.[16,18,47] This picture of the composition of the mustard oil bomb seems to be the most reliable one at present. There may be evolutionary pressure for the substrate and the enzyme to be located in neighboring cells and not randomly, so the chance for invaders to circumvent the "bomb" is minimized.

IN VIVO DEGRADATION AND TRANSPORT OF GLUCOSINOLATES

In addition to the toxic mustard-oil bomb that is dependent on tissue disruption, several experiments indicate a "safe" *in vivo* degradation of glucosinolates in intact, non-wounded plants. Perhaps the most obvious indication of this process is the release of small amounts of volatile glucosinolate hydrolysis products from intact plants of *Brassica* and *Raphanus* species.[69] However, this seems to be the only example of an investigation of completely intact plants. An *in vivo* degradation should require both intra- and intercellular transport systems. Symplastic glucosinolate transport to the myrosin cell in *Arabidopsis* is possible, as plasmodesmata have been detected between S-cells and myrosin cells, although not with high frequency (Jørgensen LB, unpublished).

In many reports that indicate *in vivo* degradation, only concentrations of glucosinolates were measured, and expansion of tissues or transport of glucosinolates were not taken into account. Therefore, we have selected a few experiments for

discussion of *in vivo* degradation. Svanem et al. extracted both glucosinolates and degradation products in a liquid nitrogen/ethanol slush and found that approximately 10% of the fed radioactive phenylethylglucosinolate was converted into isothiocyanate in *Nasturtium officinale*.[70] A low turnover of glucosinolates during germination has been reported in both *B. napus* and *Arabidopsis*.[49,71] A dramatic reduction in glucosinolate content is evident in germinating seedlings of *B. napus* (Petersen BL and Andréasson E, unpublished). This is probably a consequence of nutrient starvation because the seeds were germinated only with addition of tap water. It indicates that *B. napus* has the capacity to degrade glucosinolates efficiently. In addition to analyzing a fixed number of embryos or seedlings at every stage investigated, the content of the growth media has also been analyzed. The myrosin cell structure and MBP (myrosinase-binding protein) expression change considerably during germination.[18,33,72-74] The vacuole in the myrosin cell (the myrosin body) becomes less invaginated during germination. Glucosinolates and MBP are probably co-localized in the mature *B. napus* embryo outside the myrosin cells,[13,18] but during early germination, MBP disappears outside myrosin cells, and later the MBPs are located in the myrosin cells.[18,75] The result of James and Rossiter,[52] showing that the myrosinase activity increases during this period in *B. napus*, also indicates a dynamic nature of myrosinase and myrosin cells, which may imply that myrosin cells are involved in *in vivo* degradation of glucosinolates. Recently Bak et al.[76] and Kutz et al.[77] suggested that indole glucosinolate hydrolysis products can be precursors of auxin. This would be the clearest example of a specific use of glucosinolates from *in vivo* degradation. More generally, glucosinolates have been postulated for a long time to serve as a source of nitrogen and sulfur.

As described, glucosinolates have been shown by immunohistochemisty, cell sap analysis, and x-ray microanalysis of sulfur to be located in cells different from those that contain myrosinase. The work by Thangstad et al. suggests that some kind of cell-specific transport system may exist in myrosin cells of *B. napus* developing embryos.[14] Uptake of glucosinolates has been studied in developing embryos and leaf protoplasts from *B. napus*. The studies on embryos indicated that the uptake is carrier-mediated, and the protoplast study indicated that it is a glucosinolate/H^+ symporter mediated transport; i.e., biochemical data for a specific and active transport were reported.[78-80] The transport of glucosinolates from siliques to developing embryos is the best studied system, including biochemical characterization,[78, 79] grafting experiments,[81] reciprocal crosses[82, 83] and radioactive feeding experiments.[84, 85]

Long-distance transport of other secondary metabolites has been demonstrated, e.g., for nicotine.[86] Rosa[87] detected diurnal variations in glucosinolate content, which probably reflects transport or degradation. Lykkesfeldt and Møller[88] compared the biosynthetic capacity with the actual content of glucosinolates in different tissues of *Tropaeolum majus*; the study suggested transport from leaves to other parts of the plant where the biosynthetic capacity was low. Both glucosinolates

and desulfoglucosinolates have physiochemical properties for phloem mobility.[89] In the phloem sap of young leaves of *B. nigra*, glucosinolates have been detected in concentrations as high as 10 mM.[90] Koroleva et al. found similar concentrations in phloem exudates, but the concentration in the S-cells was approximately 10 times higher.[16] Recently, it has been shown that intact glucosinolates are transported in the phloem.[91] It was earlier proposed that glucosinolates are transported in the phloem rather than in the xylem,[89] since myrosinase was claimed to be present in the latter tissue.[43] However, a more recent study did not find evidence for myrosinase in the xylem.[18] Many of the long-distance transport analyses are based on radioactive feeding experiments, and these do not clarify the biological significance of the transport. Further experiments are needed to understand this process, e.g., *in situ* expression analysis of glucosinolate biosynthetic genes and expression-alternation studies of the transporters.

VARYING MYROSINASE DISTRIBUTION AMONG SPECIES

There are differences in the myrosinase gene family between *A. thaliana* and larger Brassicaceae members in the tribe Brassiceae, such as *B. napus* and *Sinapis alba*.[6] The difference in myrosinase expression is especially pronounced in seeds where small amounts of myrosinase are expressed in *A. thaliana*,[18,49] while in *B. napus*, members of all three myrosinase gene subfamilies are expressed. Figure 4.3 shows the specific immunohistochemical localization of myrosinase in myrosin cells in the ground tissue of *B. napus* embryos (Fig. 4.3A), while in *A. thaliana* embryos no expression was identified (Fig. 4.3B). Myrosinase in *A. thaliana* leaves is located in phloem myrosin cells (Fig. 4.3D), whereas in *B. napus* leaves, myrosinase is in myrosin cells in the ground tissue (Fig. 4.3E), as well as in the phloem (Fig. 4.3C).[18,47,74] Actually, only in the seed, not in the vegetative organs, are all three myrosinase gene subfamilies expressed in *B. napus*. This difference in myrosinase expression may be related to the size of the seeds or number of seeds produced by each individual, or to levels of biotic stress.

SUMMARY

Recent studies of *Arabidopsis* flower stalks show that the constituents of the mustard-oil bomb, namely myrosinase and glucosinolates, are stored in adjacent cells. *In vivo* degradation of glucosinolates, possibly involving myrosin cells, and the transport of glucosinolates affect the localization of the myrosinase-glucosinolate system, showing a dynamic nature that is complementary to the "static" toxic mustard oil-bomb. Large variability in expression of myrosinase among different species and organs have been detected, and this may indicate different primary roles of the myrosinase-glucosinolate system. Further reseach areas should include

characterization of transporters and proteins responsible for the *in vivo* degradation, and also localization studies of the biosynthetic genes. This knowledge will create a basis for functional and evolutionary studies of the myrosinase-glucosinolate system.

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Chapter Five

GLUCOSINOLATE HYDROLYSIS AND ITS IMPACT ON GENERALIST AND SPECIALIST INSECT HERBIVORES

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INTRODUCTION

The glucosinolate-myrosinase system is an important anti-herbivore defense in plants of the order Capparales, which includes the many agriculturally important crops of the Brassicaceae family, such as broccoli, cabbage, cauliflower, and rapeseed. Glucosinolates are sulfur-rich β-thioglucosides, while myrosinases are thioglucosidases capable of hydrolyzing glucosinolates. These two components are compartmentalized in different cells within the same tissue. However, upon tissue damage they come into contact, and glucosinolates are hydrolyzed to a suite of biologically active compounds, such as the isothiocyanates, also known as mustard oils (Fig. 5.1). Many of these hydrolysis products are toxic to a diverse range of organisms including bacteria, fungi, nematodes, and insects.[1-3]

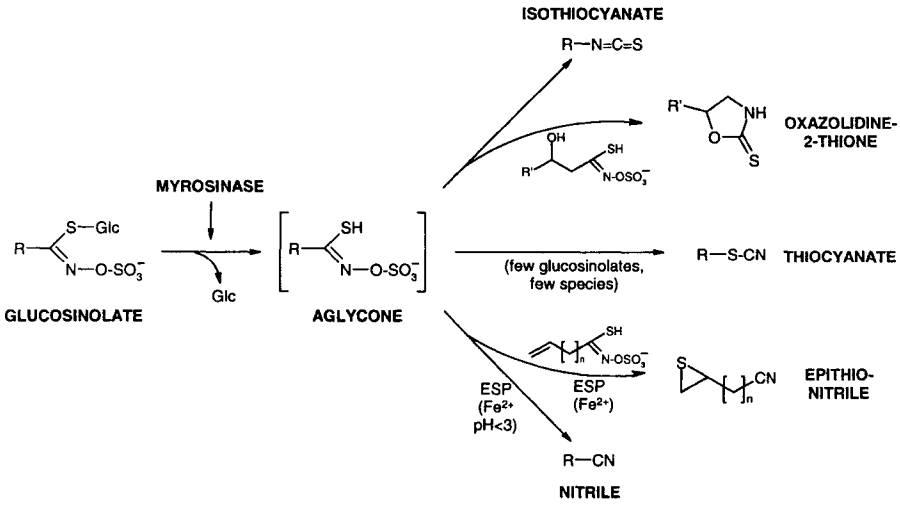

Fig. 5.1: Hydrolysis of aromatic and aliphatic glucosinolates by myrosinases. Once the glucose moiety is cleaved, the resulting aglycone rearranges into an isothiocyanate, oxazolidine-2-thione, nitrile, epithionitrile, or thiocyanate depending on the structure of the glucosinolate side chain and presence of protein factors such as epithiospecifier proteins (ESP). ESPs were originally reported as factors that lead to the formation of epithionitriles from alkenylglucosinolates upon myrosinase-catalyzed glucosinolate hydrolysis. However, the recently described ESP from *A. thaliana* also catalyzes the formation of simple nitriles from alkylglucosinolates.

That the glucosinolate-myrosinase system actually functions as a defense against certain insects has been demonstrated in a number of studies, such as those carried out recently by Li and colleagues,[4] who used *Brassica juncea* lines with high and low glucosinolate content and high and low myrosinase content in feeding tests with the southern army worm, *Spodoptera eridania*. Lines with a high glucosinolate and myrosinase content experienced much less caterpillar damage than lines with a low content of both. However, some herbivores have developed mechanisms to cope with these defenses. Specialist insects, such as the cabbage white butterflies, *Pieris rapae* and *Pieris brassicae*, are attracted to glucosinolate-containing plants. They can detect intact glucosinolates in leaves of Brassicaceae with specialized receptor cells, are stimulated to lay their eggs on these plants, and the larvae eat enormous amounts of foliage that is otherwise well defended.[5]

Despite many years of research on the role of the glucosinolate-myrosinase system in plant-insect interactions, there are still many unanswered questions concerning the biochemical bases of its effects on certain insects and ineffectiveness against others. The complexity of the biological and chemical interactions in the system are hard to dissect in experimental settings.[2] For example, artificial diet studies with glucosinolates or their hydrolysis products, although valuable, do not always allow conclusions about the toxic effects of these substances under natural conditions. If, on the one hand, intact glucosinolates are incorporated into an artificial diet, this ignores the fact that under natural conditions glucosinolates would be hydrolyzed by myrosinases upon tissue damage. If, on the other hand, isolated glucosinolate hydrolysis products are incorporated into artificial diets, the results may be misleading for at least three reasons: the hydrolysis products may react with components of the diet before ingestion, volatile hydrolysis products in the gas phase that are perceived prior to feeding may artificially influence feeding behavior, and in most cases it is not known which hydrolysis products a particular insect actually encounters after damaging a plant species. Furthermore, such studies have been limited by the availability of pure test compounds and have, therefore, been carried out with only a limited number of glucosinolates and their hydrolysis products.

In addition to artificial diet studies, many workers have tested the effects of intact glucosinolate-containing plants or plant extracts on herbivores and pathogens. Although many significant effects have been found, it has often been hard to interpret the results due to the fact that the plants tested differed from each other in many parameters besides glucosinolate and myrosinase content. The same limitation applies to certain studies in which plant chemistry had been manipulated experimentally (reviewed in ref.[6]). However, the identification of genes involved in glucosinolate biosynthesis and hydrolysis has now opened the possibility of generating plants with specific alterations in glucosinolate metabolism as tools to investigate the ecological role of the glucosinolate-myrosinase system. In fact, the first studies using near-isogenic lines or transgenic plants have already been published.[6,7]

Elucidation of the selection pressures that have driven the evolution of the glucosinolate-myrosinase system and insect counter-adaptations will likely require an interdisciplinary approach employing modern analytical techniques, plant lines whose glucosinolate metabolism has been modified, and detailed biochemical, pharmacological, and behavioral studies. In the present review, we summarize published studies showing the effects of specific glucosinolate hydrolysis products on insects and report on our own recent work on glucosinolate hydrolysis in *Arabidopsis thaliana* and its influence on the generalist herbivore *Trichoplusia ni.* In addition, some recent findings on glucosinolate detoxification by specialist insects are described.

GLUCOSINOLATE HYDROLYSIS IN PLANTS

Although there are scattered indications of glucosinolate breakdown in intact plants,[1] nearly all of our information on glucosinolate hydrolysis comes from studies on damaged plants or plant extracts. Hydrolysis of the thioglucosidic bond of glucosinolates by myrosinase[1,3,8] leads to the release of glucose and an aglucone which is unstable (Fig. 5.1). Depending on the plant species and the structure of the glucosinolate side chain, the aglycone rearranges to different products, including isothiocyanates, oxazolidine-2-thiones, nitriles, epithionitriles, and thiocyanates.

Isothiocyanates are the predominant hydrolysis products found in many species. To form isothiocyanates, the aglycone undergoes a Lossen rearrangement. The side chain migrates from the oxime carbon atom to the adjacent nitrogen atom with concerted loss of the sulfate group. However, if the glucosinolate side chain is hydroxylated at the second or third carbon atom, the resulting isothiocyanate is unstable and spontaneously cyclizes to an oxazolidine-2-thione (*e.g.* goitrin).

A major proportion of the glucosinolate hydrolysis products formed upon myrosinase cleavage in some plants are nitriles. *In vitro*, nitrile formation associated with myrosinase-catalyzed hydrolysis is enhanced at low pH (pH<3) and in the presence of ferrous ions. *In vivo*, protein factors in addition to myrosinase may be responsible for nitrile formation. If the glucosinolate side chain has a terminal double bond, the sulfur released from the thioglucosidic bond may be captured by the double bond and an epithionitrile is formed.[9] This reaction takes place only in plants that possess a protein factor known as epithiospecifier protein (ESP). ESP activities have been identified in several species of the Brassicaceae and shown to influence the outcome of the myrosinase-catalyzed hydrolysis reaction although they have no hydrolytic activity by themselves.[10-12] The mechanism by which ESPs promote epithionitrile formation is not known.

Thiocyanates are apparently only formed from three glucosinolates, benzyl-, allyl-, and 4-methylsulfinylbutyl-glucosinolate.[13,14] The ability to produce thiocyanates seems to be restricted to just a few plant species, and, like epithionitrile formation, may require the presence of specific protein factors.

Glucosinolates are grouped based on the chemical nature of their side-chain (R-group, Fig. 5.1): aliphatic, benzylic, or indole. The hydrolysis of indole glucosinolates is generally different from the hydrolysis of aliphatic and benzylic glucosinolates. While nitriles are known to be formed from the breakdown of indole glucosinolates, the isothiocyanates derived from indole glucosinolates seem to be unstable and are converted to several other metabolites, including indole-3-carbinol and indole ascorbigen with release of a thiocyanate ion.[15,16] Recent data obtained with *Brassica oleracea* suggest that thiocyanate ions may be methylated by thiol-methyltransferases to yield volatile methylthiocyanate, one of several volatile sulfur compounds detected in the headspace of species of the Brassicaceae.[17,18]

In summary, the outcome of glucosinolate hydrolysis *in vivo* is influenced by both the chemical structure of the side-chain and the presence of particular protein factors, reactants (ascorbate) and cofactors (ferrous ions) within individual plant species. However, there is still much to be learned about the mechanisms of this process and the occurrence of different glucosinolate hydrolysis products in plant species. The diversity of hydrolysis products further amplifies the diversity of parent glucosinolate structures present (Fig. 5.1), and may have evolved in response to the selection pressure of a multitude of different enemies. In the next section, we review studies on the biological activities of isolated glucosinolate hydrolysis products on insects. Such investigations provide essential information for understanding the ecological significance of the glucosinolate-myrosinase system.

EFFECTS OF ISOLATED GLUCOSINOLATE HYDROLYSIS PRODUCTS ON INSECTS

Toxic and Deterrent Effects on Insects

Numerous studies have shown that glucosinolate hydrolysis products or extracts of glucosinolate-containing plants are toxic to a wide range of organisms besides insects, including bacteria, fungi, nematodes, and mammals.[1,3,19-24] Many of these investigations were motivated by concerns associated with the toxic effects of glucosinolate-containing animal feed and by the cancer-preventing activity of glucosinolate-containing vegetables in humans, as well as by the desire to use plant tissues as biopesticides. While these investigations bear testimony to the broad range of biological activities associated with glucosinolate hydrolysis products, due to the large volume of such reports, we limit the present review to studies that employed isolated glucosinolate hydrolysis products and that tested their effect on insects directly. However, we also include non-insect studies where the effects of different hydrolysis products of a single glucosinolate were compared.

The glucosinolate hydrolysis products most frequently demonstrated to be toxic to insects are the isothiocyanates (Table 5.1). In fact, isothiocyanates have been

Table 5.1: Examples of studies showing toxic effects of isolated glucosinolate-hydrolysis products on insects. NCS, isothiocyanate; SCN, thiocyanate; CN, cyanide (= nitrile).

compound	insect species	effect	comments	ref.
2-phenylethyl-NCS	vinegar fly (*Drosophila melanogaster*), housefly (*Musca domestica*), pea aphid (*Macrosiphum pisi*), spider mite (*Tetranychus atlanticus*)	toxic as fumigant and upon contact	isolated by bioassay guided fractionation of turnip	21
benzyl-NCS	Hawaiian fruit flies (*Dacus dosalis, D. cucurbitae, Ceratitis capitata*)	toxic to eggs and first instars upon contact	isolated as insecticidal compound from *Carica papaya*	57
benzyl-NCS allyl-NCS 2-phenylethyl-NCS benzyl-SCN	fall army worm (*Spodoptera frugipera*), cabbage looper (*Trichoplusia ni*), velvet bean caterpillar (*Anticarsia gemmatalis*)	toxic upon feeding (artificial diet)	R-NCSs toxic in same concentration range, benzyl-SCN ca. 5-10x less toxic than R-NCSs	29,49
3-methoxy-benzyl-NCS	fall army worm (*Spodoptera frugipera*), European corn borer (*Ostrinia nubilalis*)	toxic upon feeding (artificial diet)	isolated by bioassay-guided fractionation of *Limnanthes alba*, *S. frugipera* almost 3x more sensitive than *O. nubilalis*	58
allyl-NCS allyl-CN goitrin indoleacetonitrile indole-3-carbinol	locust (*Schistocerca gregaria*)	feeding deterrent (sucrose-impregnated filter paper)	allyl-CN 100x less deterrent than allyl-NCS, goitrin and indole derivatives 10x less deterrent than allyl-NCS; possible loss of volatile compounds by evaporation during drying not accounted for	59
allyl-NCS allyl-CN allyl-SCN	lesser grain borer (*Rhizopertha dominica*), housefly (*M. domestica*)	toxic as fumigant	toxicity: allyl-SCN≥allyl-NCS>allyl-CN (effective concentrations in air calculated based on 100 % volatility)	25

compound	insect species	effect	comments	ref.
benzyl-NCS benzyl-CN benzyl-SCN	lesser grain borer (*Rhizopertha dominica*), housefly (*M. domestica*)	toxic as fumigant	toxicity to *R. dominica*: benzyl-CN≥benzyl-SCN>benzyl-NCS toxicity to *M. domestica*: benzyl-CN≥benzyl-NCS>benzyl-SCN (effective concentrations in air calculated based on 100 % volatility)	25
allyl-NCS benzyl-NCS 2-phenylethyl-NCS	black wine weevil (*Otiorhynchus sulcatus*)	toxic to eggs upon contact	toxicity: 2-phenylethyl-NCS≥benzyl-NCS>allyl-NCS	60
2(*S*)-hydroxy-3-butenyl-CN	housefly (*M. domestica*)	toxic upon contact	isolated by bioassay guided fractionation of *Crambe abyssinica* seed meal, (R)-enantiomer less toxic	31
allyl-NCS	diamondback moth (*Plutella xylostella*), southern army worm (*Spodoptera eridania*)	toxic upon feeding (artificial diet)	crucifer specialist *P. xylostella* as sensitive as generalist *S. eridania*	4

frequently shown to be responsible for the biological activity of extracts from glucosinolate-containing plants to a range of different organisms.[21-24] For insects, isothiocyanates have been demonstrated to be toxic in the gas phase, upon contact, and after ingestion, sometimes in the same concentration range as commercial insecticides. Among other hydrolysis products, nitriles and thiocyanates have also evidenced toxicity to insects, but nothing is known about possible toxic effects of glucosinolate-derived epithionitriles and oxazolidine-2-thiones. Since oxazolidine-2-thiones such as goitrin are toxic to many mammals by interfering with the metabolism of the thyroid gland, they may function as defenses against mammalian herbivores.

Few studies have directly compared the effects of different hydrolysis products derived from the same glucosinolate. Such comparative studies with insects are listed in Tables 5.1-3, and comparative studies with other organisms in Table 5.4. In many cases, isothiocyanates seem to have greater biological activity than nitriles, thiocyanates, and oxazolidine-2-thiones. However, comparisons are complicated by differences among the hydrolysis products in physico-chemical properties, such as volatility. If two hydrolysis products differ significantly in volatility, for example,

then quantitative comparisons of their toxic effects as fumigants[25] may not be justified unless the actual concentrations in the gas phase have been measured. Comparisons among hydrolysis products in their effects on feeding in artificial diets are also complicated by the possibility that isothiocyanates may react with components of the diet, such as proteins. The relative lack of data on the bioactivity of nitriles and epithionitriles is likely due to the fact that most are not commercially available, and thus have not been frequently tested with probable target organisms.

Little is known about the mechanisms by which glucosinolate hydrolysis products exert their toxicity to insects. Isothiocyanates react with amino groups of proteins and cleave disulfide bonds *in vitro*.[26,27] This property, which is used in the Edman degradation of proteins for amino acid sequence analysis, may, at least in part, account for the general toxicity of isothiocyanates. The release of HCN upon the reaction of benzylthiocyanate with glutathione, catalyzed by glutathione-S-transferase in the insect midgut, has been suggested as the basis of toxicity of benzyl-thiocyanate to different insects.[28,29] A similar mechanism is assumed to be responsible for the toxicity of nitriles to mammals.[28,30] However, the difference in potency between 2(*S*)-hydroxy-3-butenyl-cyanide and its 2(*R*)-enantiomer indicates that the mechanism of nitrile toxicity is still not fully understood.[31]

In the majority of studies discussed here, the toxic effects of glucosinolate hydrolysis products were assessed against generalist insect herbivores. Little is known about the effects of glucosinolate hydrolysis products on insects specialized on Brassicaceae. An exception is the diamond back moth (*Plutella xylostella*), whose larvae are highly sensitive to allyl-isothiocyanate, but can nevertheless feed on plants with high allyl-glucosinolate and myrosinase levels.[4] This raises the question of how this species circumvents the toxic effects of isothiocyanates when feeding on its host plants (discussed later in this chapter).

Attraction of Specialist Herbivores and Parasitoids

Glucosinolate hydrolysis products not only poison and deter insect herbivores, but also serve as attractants to insects that specialize on Brassicaceae species. Both isothiocyanates and nitriles have been shown to be involved in attracting adults of certain insects specialized on Brassicaceae to their host plants (Table 5.2). Moreover, the responses of some of these insects seem to be specific to particular isothiocyanates and nitriles. For example, the cabbage seed weevil, *Ceutorhynchus assimilis*, is attracted to 3-butenyl- and 4-pentenyl-isothiocyanate, but not to 2-phenylethyl-isothiocyanate.[32,33] However, the response to the mixture of all three isothiocyanates is much greater than the response to the individual compounds at equivalent concentrations. The sensitivity to specific mixtures may help specialist feeders recognize different Brassicaceae with different glucosinolate compositions. The differential response of *C. assimilis* (and of the cabbage stem flea beetle, *Psylliodes chrysocephala*) to different isothiocyanates has been shown to be

reflected at the sensory level by gas chromatography coupled with an electroantennogram [34] and analysis of single olfactory cells.[35] While isothiocyanates and nitriles are involved in the host finding and long distance recognition of many Brassicaceae specialists, once specialists such as *Pieris rapae*, *Delia radicum*, and *Phyllotreta nemorum* arrive on the plant, intact glucosinolates serve as major oviposition and feeding stimulants .[5,36,37]

Insects that parasitize herbivores on Brassicaceae are also attracted by glucosinolate hydrolysis products. Since hydrolysis products are released after plant damage, this attraction serves to guide parasitoids to plants on which their hosts are likely to be found. Adult females of several kinds of wasps whose larvae are endoparasitoids on specialist, Brassicaceae-feeding aphid, cutworm and midge species were attracted to certain isothiocyanates, in particular allyl-isothiocyanate, in laboratory and field experiments (Table 5.3). Other types of glucosinolate hydrolysis products (*e.g.,* nitriles) have not been tested in such systems. Extensive investigations of *Cotesia rubecula*, an endoparasitoid of larvae of the cabbage white, *Pieris rapae*, a specialist lepidopteran feeding on Brassicaceae, have shown that volatile blends from caterpillar-damaged plants are involved in attracting adult *C. rubecula* females to their hosts for oviposition.[38] Both nitriles and isothiocyanates have been identified in these blends along with terpenoids and fatty acid derivatives.[39,40] In addition to volatiles, *Cotesia glomerata* uses contact cues from frass, silk, and herbivore-damaged leaf-tissue to determine the presence of its host *Pieris brassicae*.[41]

AN *ARABIDOPSIS THALIANA* EPITHIOSPECIFIER PROTEIN (ESP) ALTERS GLUCOSINOLATE HYDROLYSIS

Natural Variation in Glucosinolate Hydrolysis Products Among A. thaliana *Ecotypes*

The previous section has demonstrated the value of tests with isolated glucosinolate hydrolysis products in establishing the effects of specific compounds on insect herbivores. However, such tests are necessarily artificial in the absence of the other components of the plant. In addition, it is very difficult to simulate the actual release rate of glucosinolate hydrolysis products from damaged plants in an experimental setting. The identification of plant genes that control the formation of glucosinolate hydrolysis products will facilitate the manipulation of levels and types of these products formed in plants and allow their effects on insects to be tested under realistic conditions. Thus, we sought genes regulating glucosinolate hydrolysis in *A. thaliana*, which has already proved to be a useful model plant for identifying genes and enzymes involved in glucosinolate biosynthesis.[42]

Table 5.2: Examples of studies showing attraction of insects specialized on Brassicaceae by isolated glucosinolate hydrolysis products. NCS, isothiocyanate; CN, cyanide (= nitrile).

compound	species; experimental mode	effect	comments	ref.
allyl-NCS phenylethyl-NCS phenyl-NCS	flea beetles (*Phyllotreta*, sp.), weevils (*Ceutorhynchus* sp.); 'attracting tube-trap' in field	attractive less attractive not attractive	 not naturally occuring	61
allyl-NCS	turnip root fly (*Delia floralis*); laboratory tests of turnip pieces with added test compound	attractive		62
allyl-NCS	cabbage root fly (*Delia brassicae*); laboratory tests of artificial substrates with added test compound	attractive	R-NCS alone did not stimulate oviposition	63
allyl-NCS	cabbage white (*Pieris rapae*, *P. brassicae*), vegetable weevil (*Listroderes obliquus*), cabbage stem flea beetle (*Psylliodes chrysocephala*); experimental mode not indicated	attractive		ref. cite d in 64
ethyl-NCS, allyl-NCS, benzyl-NCS synthetic R-NCSs	cabbage root fly (*Delia brassicae*); yellow and black water traps in field	attractive	no indications for synergism, olfactory cues sufficient for locating odor source	65
3-butenyl-NCS 4-pentenyl-NCS 2-phenylethyl-NCS allyl-NCS	cabbage seed weevil (*Ceutorhynchus assimilis*); yellow water traps in field experiments	attractive during immigration to host crop not attractive	mixture of all 4 R-NCSs attractive during immigration, but not during host colonization	66
benzyl-CN benzyl-alcohol	cabbage seed weevil (*Ceutorhynchus assimilis*); yellow water traps in field experiments	attractive during host colonization		66
3-butenyl-NCS 4-pentenyl-NCS 2-phenylethyl-NCS	cabbage seed weevil (*Ceutorhynchus assimilis*); linear track olfactometer, wind-tunnel; linear track olfactometer	attractive not attractive	synergism: mixture of all three R-NCSs resulted in response at 1/10 the amount for individual R-NCSs	32 33

compound	species; experimental mode	effect	comments	ref.
3-butenyl-CN 4-pentenyl-CN benzyl-CN 2-phenylethyl-CN 2-phenylethanol indole, goitrin	cabbage seed weevil (*Ceutorhynchus assimilis*); linear track olfactometer	attractive not attractive	synergistic effects of R-NCSs and benzyl-CN?	67
allyl-NCS ethyl-NCS, benzyl-NCS, 3-methylthio-propyl-NCS, butyl-NCS 2-phenylethyl-NCS methyl-CN, allyl-CN, phenyl-CN, benzyl-CN	flea beetles (*Phyllotreta cruciferae, Phyllotreta striola*); yellow plastic boll traps in field; 'choice-trap' in field	most attractive attractive low attraction low/no attraction	concentration-dependent effect poorly reproducible	68,69

Table 5.3: Examples of studies showing attraction of parasitoids of insects specialized on Brassicaceae by isolated glucosinolate hydrolysis products. NCS, isothiocyanate ; CN, cyanide (= nitrile).

compound	species; experimental mode	effect	ref.
allyl-NCS	*Diaeretiella rapae* (parasitoid on cabbage aphid, *Brevicoryne brassicae*); direct application of allyl-ITC on plant	higher parasitation rate	70
allyl-NCS propyl-NCS, ethyl-NCS, 2-phenyl-ethyl-NCS	*Meteorus leviventris* (parasitic wasp on crucifer-feeding cutworms); yellow plastic boll traps in field	attractive not attractive	71
allyl-NCS phenylethyl-NCS	brassica pod midge (*Dasineura brassicae*), parasitoids of *D. brassicae, Platygaster subuliformis, Omphale clypealis*; selective flight traps in field	attractive only to *D. brassicae* and females of *O. clypealis* attractive only to *P. subuliformis*	72

Table 5.4: Examples of studies comparing effects of different hydrolysis products derived from the same glucosinolate. NCS, isothiocyanate; CN, cyanide (= nitrile).

compounds tested	activity tested	comparison of effects	comments	ref.
allyl-NCS vs. allyl-CN	growth inhibition of downy mildew (*Peronospora parasitica*)	R-NCS highly active, R-CN not active		73
indoleacetonitrile vs. indole-3-carbinol	growth inhibition of plant-pathogenic fungus (*Leptosphaeria maculans*)	indole-3-carbinol more inhibitory	experiments with aliphatic glucosinolates in the presence of myrosinase: stronger growth inhibition at pH7 (R-NCS formation) than at pH4 (nitrile formation)	74
indoleacetonitrile vs. indole-3-carbinol	growth inhibition of plant pathogenic bacterium (*Erwinia carotovora*)	indoleacetonitrile more inhibitory		75
allyl-NCS vs. allyl-CN, 2-phenylethyl-NCS vs. 2-phenylethyl-CN	inhibition of soil nitrifying bacteria	R-NCSs more inhibitory	measured as decrease in NO_3^- nitrogen in soil	76
allyl-NCS vs. allyl-CN	toxicity to nematode (*Caenorhabditis elegans*)	allyl-ITC 400x more toxic		77
2-hydroxy-3,4-epithiobutyl-CN vs. 2-hydroxy-3-butenyl-CN vs. goitrin	phytotoxic activity to green lettuce (*Lactuca sativa*)	2-hydroxy-3,4-epithio-butyl-CN 5-10x more toxic (inhibition of germination and root growth) than 2-hydroxy-3-butenyl-CN and goitrin; 2-hydroxy-3-butenyl-CN inhibits germination, goitrin inhibits root growth		78
3,4-epithiobutyl-CN vs. butyl-CN	toxic effects on rats after oral administration	both toxic with different symptoms	indications for different mechanisms of toxicity	30

compounds tested	activity tested	comparison of effects	comments	ref.
goitrin vs. 2(S) - hydroxy-3-butenyl-CN, R-NCSs vs. R-CNs of 3-methylsulfinylpropyl-, 4-methylsulfinylbutyl-, benzyl-, p-hydroxy-benzyl-glucosinolate	antiproliferative activity to human erythroleukemic K562 cells	goitrin 5x more active than corresponding nitrile, R-NCSs 7-280x more active than corresponding nitriles, exception: p-hydroxy-benzyl-NCS 3x less active than corresponding nitrile		79
4-methylsulfinylbutyl-NCS vs. 4-methyl-sulfinylbutyl-CN	induction of phase II detoxification enzymes in rats and mouse hepatoma cells	rats: R-NCS active, R-CN not active, mouse hepatoma cells: R-NCS 800x more active	enzymes tested: quinone reductase, glutathione S-transferase	80
4-methylsulfinylbutyl-NCS vs. 4-methylsulfinylbutyl-CN	induction of phase II detoxification enzymes in a human hepatoma cell line	R-NCS 2-5x more active than R-CN	enzymes tested: glutathione S-transferase, UDP-glucu-ronosyl transferase; R-NCS-glutathione-conjugate as active as R-NCS on glutathione S-transferase	81

We surveyed the formation of glucosinolate hydrolysis products in *A. thaliana* and found that ecotypes of this species not only vary in their glucosinolate content and composition,[43] but also in the nature of glucosinolate hydrolysis products formed on leaf damage. Analysis of dichloromethane extracts of aqueous leaf macerates of 122 *A. thaliana* ecotypes by gas chromatography-mass spectrometry (GC-MS) and gas chromatography with flame ionization detection (GC-FID) showed that the most abundant aliphatic glucosinolates are mainly hydrolyzed to either isothiocyanates or nitriles.[44] Figure 5.2a shows the GC-FID traces obtained from extracts of leaf macerates of the ecotypes Monte Tosso (Mr-0) and Eifel (Ei-2). In both ecotypes, allyl-glucosinolate is the major glucosinolate in leaves. However, in Mr-0, allyl-glucosinolate is hydrolyzed mainly to the corresponding isothiocyanate, while in Ei-2 hydrolysis leads to the formation of the corresponding epithionitrile

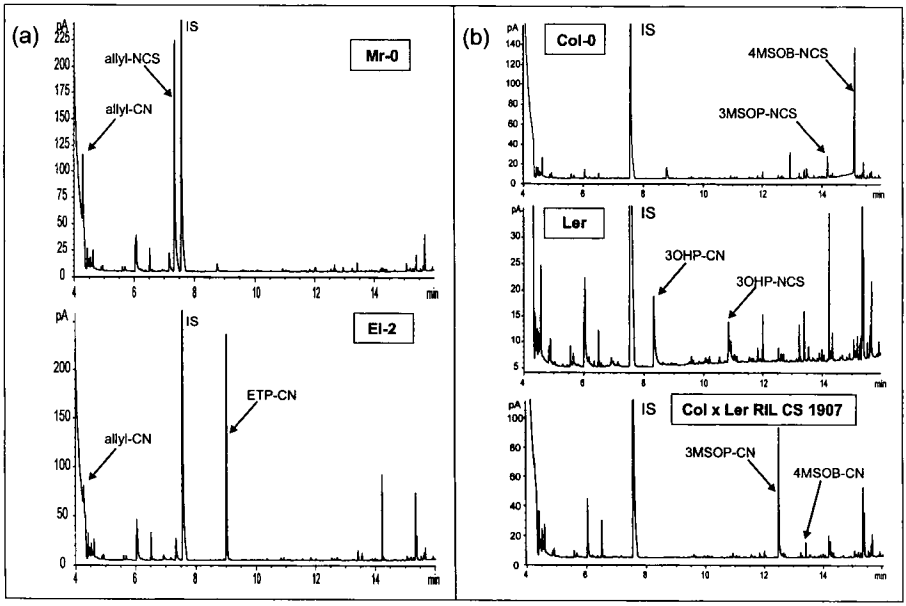

Fig. 5.2: Natural variation in glucosinolate hydrolysis products in *A. thaliana* ecotypes.[44] (a) Allyl-glucosinolate is mainly hydrolyzed into the corresponding isothiocyanate in Mr-0, but into the epithionitrile in Ei-2. (b) The main glucosinolates in Col-0 (4-methylsulfinylbutyl- and 3-methylsulfinylpropyl-glucosinolate) are hydrolyzed into the corresponding isothiocyanates. In L*er*, the major glucosinolate (3-hydroxypropyl-glucosinolate) is hydrolyzed into the corresponding nitrile. The Col-0 × L*er* recombinant inbred line (RIL) CS 1907 accumulates the Col-0-glucosinolates, but their hydrolysis is controlled by the L*er*-ESP-allele (nitriles are formed). Depicted are GC-FID traces of dichloromethane extracts of aqueous leaf macerates. Compounds were identified by mass spectrometry and retention time relative to authentic standards. -NCS, isothiocyanate; -CN, cyanide (=nitrile); ETP, 2,3-epithiopropyl; 4MSOB, 4-methylsulfinylbutyl; 3MSOP, 3-methylsulfinylpropyl; 3OHP, 3-hydroxypropyl; IS, internal standard.

(2,3-epithiopropyl-cyanide=3,4-epithiobutyl-nitrile). Ecotypes with alkyl-glucosinolates or hydroxyalkyl-glucosinolates as their major compounds also produced either isothiocyanates or the corresponding nitriles as the principal hydrolysis products (Fig. 5.2b).

Identification of A. thaliana *ESP*

The variation in hydrolysis product formation among *A. thaliana* ecotypes was used to map the location of genes controlling this process. A mapping population was chosen whose parents, the ecotypes Columbia (Col-0) and Landsberg *erecta* (L*er*), differ in their major glucosinolates and in the type of hydrolysis products formed. Col-0 accumulates 4-methylsulfinylbutyl-glucosinolate in the leaves, while the main leaf glucosinolate in L*er* is 3-hydroxypropyl-glucosinolate. Upon hydrolysis, Col-0 forms mainly isothiocyanates, while L*er* produces mainly nitriles (Fig. 5.2b). In crosses of the two ecotypes, the progeny segregate with respect to both glucosinolate composition and hydrolysis product formation. Ninety six recombinant inbred lines derived from a Col-0 × L*er* cross (obtained from the Arabidopsis Biological Resource Center) were used for mapping quantitative trait loci (QTL)[45] that control the outcome of the hydrolysis reaction. The ability to form nitriles co-segregated most closely with the genetic marker *nga280* on chromosome I (Fig. 5.3a). Lines that had the L*er*-allele at this locus produced mainly nitriles, while those with Col-0 alleles produced mainly isothiocyanates.[44]

By scanning the *A. thaliana* genomic sequence in close proximity to marker *nga280*, a gene was found that encoded a protein with 80 % amino acid identity to a partial sequence of an epithiospecifier protein (ESP) from *Brassica napus*.[46] No other sequence in the entire *A. thaliana* genome was more similar to the *B. napus* ESP sequence. Therefore, it seemed likely that this gene encoded an *A. thaliana* ESP that could be responsible for the difference in hydrolysis product formation between the Col-0 and L*er* ecotypes. In fact, in the recombinant inbred lines used for mapping, the expression of the transcript of this putative *A. thaliana* ESP, as documented by RT-PCR, correlated perfectly with the ability to form nitriles. To prove the catalytic properties of the protein, the cDNA of the *A. thaliana* ESP was expressed in *Escherichia coli*.[44] The *A. thaliana* ESP was able to catalyze the formation of epithionitriles from alkenylglucosinolates and of simple nitriles from alkylglucosinolates. Like other known ESPs, the protein does not catalyze glucosinolate hydrolysis by itself, but requires myrosinase to mediate nitrile formation. Other glucosinolate-containing plants that produce nitriles when damaged probably possess proteins similar to the *A. thaliana* ESP.

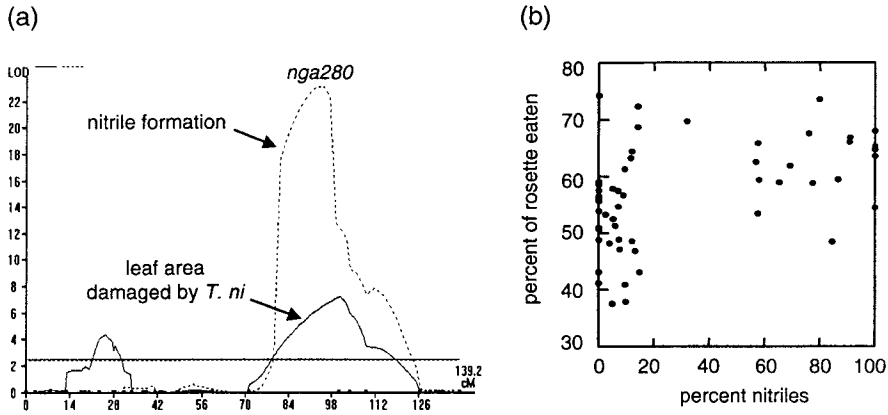

Fig. 5.3: Correlation between nitrile formation and herbivory by *Trichoplusia ni* in 96 *A. thaliana* Col-0 × L*er* recombinant inbred lines.[44] (a) The major quantitative trait loci controlling nitrile formation and *T. ni* herbivory on chromosome I are virtually identical. (b) Larvae of *T. ni* feed more on lines that produce nitriles than lines that produce isothiocyanates ($n = 56$, $r_G = 0.273$, $p < 0.001$).

Influence of ESP Activity on Trichoplusia ni *Herbivory*

Identification of an *A. thaliana* gene controlling the outcome of glucosinolate hydrolysis now permitted a direct assessment of the relative effects of isothiocyanates versus nitriles on herbivores in the context of the whole plant. We allowed first instar larvae of the generalist lepidopteran herbivore *Trichoplusia ni* (cabbage looper) to feed on rosettes of 4-week-old plants representing the same recombinant inbred lines used for QTL mapping of nitrile formation.[47] After 48 h, the leaf area that had been removed by the caterpillars was measured. The extent of caterpillar damage correlated directly with the ability of plants to produce nitriles upon glucosinolate hydrolysis (Fig. 5.3b).[44] Moreover, a significant QTL associated with feeding of *T. ni* on these lines [44,48] was found to overlap closely with the QTL for nitrile formation (Fig. 5.3a). In other words, the larvae fed more on lines that expressed ESP and, therefore, produced nitriles on glucosinolate hydrolysis than on lines that produced isothiocyanates due to a lack of ESP activity. Based on the literature reviewed above (Table 5.1), this outcome appears to be due to the higher

toxicity or deterrency of isothiocyanates compared to nitriles, but direct experimental evidence of this conclusion for *T. ni* still needs to be provided.

The ecological role of ESP and nitrile production in *A. thaliana* is not easy to understand. Why would a plant produce a protein whose activity provides less protection against herbivory? Several scenarios can be advanced in which a plant may benefit from producing nitriles instead of isothiocyanates, although, as discussed above, it is not easy to make firm generalizations about the effects of glucosinolate hydrolysis products on insects from the data currently available. (1) Epithionitriles (nitriles derived from glucosinolates with a terminal double bond) may be more toxic than the simple nitriles derived from the hydrolysis of methylsulfinylalkyl-glucosinolates formed in the *A. thaliana* lines tested with *T. ni* in the study described above. Epithionitriles contain an additional sulfur atom as part of a reactive three-membered ring that may account for additional biological activity. (2) The production of nitriles instead of isothiocyanates may also be advantageous if pressure from specialist herbivores is high. From Table 5.2, it appears that isothiocyanates have been more frequently shown to be attractants for specialist herbivores than nitriles. Thus, the lack of isothiocyanate production may allow a plant to become less apparent to certain herbivores. (3) Nitriles may also be formed because they have important functions in defense against pathogens and non-insect herbivores or in attraction of herbivore parasitoids and predators, roles that have not yet been studied. Further investigations to examine the activity of nitriles and epithionitriles on herbivores and herbivore enemies are needed to clarify their role in plant defense.

HOW INSECTS OVERCOME THE GLUCOSINOLATE-MYROSINASE SYSTEM

Despite their toxic potential, certain insect herbivore species use glucosinolate-containing plants as their major or sole hosts, and may even employ glucosinolates or their hydrolysis products in host location.[1,2] These insects must have developed the ability to circumvent the toxic effects of glucosinolate hydrolysis products, but, until recently, little was known about mechanisms that enable herbivores to overcome the glucosinolate-myrosinase system. These mechanisms may depend on the proportion of glucosinolate-containing plant species in the diet. Certain generalist insect herbivores that feed on a variety of species including Brassicaceae may have the capacity to detoxify isothiocyanates to a certain extent. Conjugation with glutathione by glutathione-S-transferases has been suggested as one route of isothiocyanate metabolism[29,49-51] since glutathione-S-transferase activity is induced in some insects upon ingestion of isothiocyanates or glucosinolates. While the reaction of allyl-isothiocyanate with reduced glutathione has been studied in

detail,[26] none of the insect glutathione-S-transferases characterized has been shown yet to actually metabolize glucosinolate-derived isothiocyanates.

Specialist herbivores of glucosinolate-containing plants may have high levels of isothiocyanate detoxification activity or employ other strategies to prevent the initial release of toxic hydrolysis products from the ingested plant tissue. An example of the latter strategy has recently been discovered in a lepidopteran specializing on the Brassicaceae.[52] Larvae of the diamond-back moth, *Plutella xylostella*, possess a sulfatase in their gut that hydrolyzes glucosinolates to desulfated derivatives that are no longer substrates for myrosinases. This blocks the formation of any toxic glucosinolate hydrolysis products. The sulfatase activity is apparently high enough to desulfate the ingested glucosinolates faster than the ingested plant myrosinases can convert them into toxic products. This detoxification mechanism is not common to all lepidopteran species that feed on Brassicaceae, since sulfatase activity was not detectable in the larvae of several other lepidopteran herbivores that can feed on Brassicaceae, including *Spodoptera exigua*, *T. ni*, and *P. rapae*.[52] Several reports on sequestration of intact glucosinolates by certain insects that have specialized on Brassicaceae[53-55] suggest that these insects must be able to avoid glucosinolate hydrolysis when ingesting plant tissue, and that they may use glucosinolates in combination with endogenous thioglucosidases to defend themselves against predators.

SUMMARY

Since the biological activities of glucosinolates arise principally from their hydrolysis products, understanding the ecological roles of glucosinolates in plant-herbivore interactions requires a detailed knowledge of the formation, occurrence, comparative activities and mode of action of their hydrolysis products. However, the reactivity and volatility of glucosinolate hydrolysis products and their limited commercial availability has made them difficult to study. In this review, we have summarized the results of a number of investigations carried out in the past few decades that assessed the effects of isolated glucosinolate hydrolysis products on insect herbivores.

The best studied hydrolysis products, the isothiocyanates, have frequently been shown to be toxic or deterrent to insects, and so would appear to serve as potent defenses against herbivory. Studies comparing the toxicity of various hydrolysis products suggest that isothiocyanates are more toxic than their corresponding nitriles, but most of this work has been carried out with microbes, nematodes, and mammals, rather than insects. More comparative studies need to be undertaken in which the full range of hydrolysis products, including epithionitriles, thiocyanates, and oxazolidine-

Further studies on the mechanism of action of the various hydrolysis products would also shed more light on their defensive function. Isothiocyanates are known to react readily with amino groups *in vitro*, and nitriles and thiocyanates are thought to release hydrogen cyanide, but much more research needs to be done to elucidate the bases of their toxicity to insects. Knowledge of the general toxicity of glucosinolate-derived compounds to insects can also be achieved by trying to determine how herbivores that specialize on glucosinolate-containing plants manage to feed without any apparent ill effects. Specialist herbivores may avoid the effects of glucosinolate hydrolysis products by detoxifying them, redirecting hydrolysis to less harmful products, or by inhibiting the hydrolysis reaction. Specialist herbivores on glucosinolate-containing plants may also employ glucosinolate hydrolysis products to find their hosts and may even sequester glucosinolates for their own defense.

Further progress in understanding the function of glucosinolate hydrolysis products in plant-herbivore interactions would be aided by studies with intact plants in which hydrolysis products have been specifically altered by genetic engineering or traditional breeding methods. We have already seen that *A. thaliana* lines containing the epithiospecifier protein form nitriles rather than isothiocyanates on glucosinolate hydrolysis. Thus, comparison of lines with and without ESP has allowed the relative impact of these different hydrolysis products to be evaluated. Initial results indicate that isothiocyanates are more toxic, but further testing is required in different glucosinolate backgrounds.

Another way to engineer changes in glucosinolate hydrolysis products is to manipulate the composition of the parent glucosinolates in a given plant. As an example, near-isogenic *Brassica napus* and *B. oleracea* lines with allelic variation at a single locus controlling glucosinolate chain-length were used to investigate the influence of volatile glucosinolate hydrolysis products of different chain-lengths on attraction of the cabbage aphid parasitoid, *Diaeretiella rapae*. Lines with high levels of 3-butenyl-glucosinolate were more attractive to *D. rapae* under field conditions and in olfactometer tests than lines with high levels of 2-propenyl-glucosinolate (and only low amounts of 3-butenyl-glucosinolate).[7] However, evidence for the involvement of the particular hydrolysis products is lacking.

Modification of the last steps of glucosinolate biosynthesis, such as altering the hydroxylation pattern or desaturation of the glucosinolate side chain, can be especially useful in altering the type of hydrolysis products that can be formed. As an example, conversion of 4-methylsulfinylbutyl-glucosinolate to 3-butenyl-glucosinolate introduces a double bond in the side chain.[56] This changes the profile of hydrolysis products from predominantly isothiocyanates and nitriles to epithionitriles (if the plant expresses a functional ESP). Further modification of 3-butenyl-glucosinolate to its 2-hydroxy-derivative introduces a hydroxyl group at carbon 2 of the side chain. This will result in the cyclization of the incipient isothiocyanate to the oxazolidine-2-thione, goitrin, instead. We have recently identified *A. thaliana* genes encoding 2-oxoglutarate-depending dioxygenases that

catalyze both of these reactions.[56] Transgenic plants with altered expression levels of these and other biosynthetic enzymes represent ideal experimental subjects for investigating the impact of different glucosinolate hydrolysis products on the interaction of plants with insects at the whole plant level. There is still much to be learned about the biological roles of the hydrolysis products. Fortunately, the rapid progress being made in identification of genes controlling the metabolism of glucosinolates in *A. thaliana*[42] provide a steady supply of valuable material for making genetically modified plants that can be used in new experiments.

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Chapter Six

A NOVEL MYROSINASE-GLUCOSINOLATE DEFENSE SYSTEM IN CRUCIFEROUS SPECIALIST APHIDS

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INTRODUCTION

Secondary metabolites in plants are thought to function as deterrents to nonadapted herbivores, while adapted herbivores have developed mechanisms of detoxification or even utilize such compounds in their own defense.[1] All cruciferous plants contain glucosinolates, and recent developments in *Arabidopsis* genomics has enabled the complex secondary metabolite biosynthetic pathway to be elucidated.[2] Glucosinolates and their degradation products[3] are responsible for the characteristic taste and odor of crops such as horseradish, cabbage, mustard, and broccoli (isothiocyanates are responsible for the 'bite' and pungency) and, therefore, in these crops the glucosinolate content is valued. There is increasing interest in the dietary role of certain glucosinolates and their breakdown products in the prevention of cancer. It is widely accepted that glucosinolates play a role in plant defense against pathogens and insect pests, although glucosinolates may also have other roles in plant metabolism.[3]

The enzyme responsible for the hydrolysis of glucosinolates is known as myrosinase (E.C. number 3.2.3.1, also known as; β-thioglucosidase, β-thioglucoside glucohydrolase). The enzyme mediated hydrolysis of glucosinolates leads to a labile aglycone, which rapidly undergoes spontaneous rearrangement, eliminating sulphur, to yield a variety of toxic metabolites such as isothiocyanates, thiocyanates, cyanoepithioalkanes, and nitriles (Fig. 6.1). The myrosinases that have been most extensively studied so far are without doubt those from cruciferous plants, followed by the microbial enzymes. A distinctive feature of plant myrosinase is its activation by ascorbate,[4] and for a review of myrosinases prior to 1983 (a historical perspective) readers are referred to Rossiter[5] and in more recent years Bones and Rossiter[3] and Rask *et al.*[6]

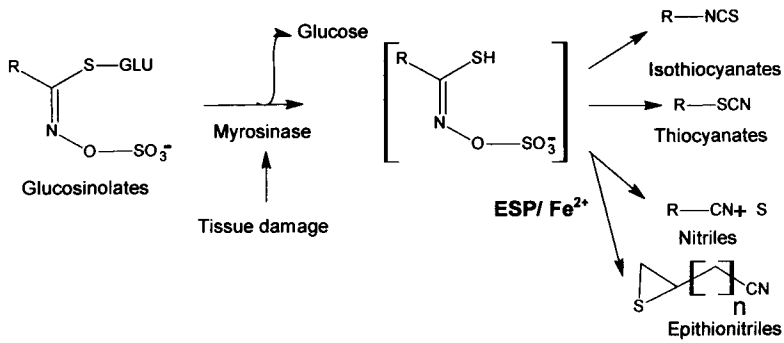

Figure 6.1: General scheme of glucosinolate hydrolysis.

The reaction products depend on pH and other factors such as the presence of ferrous ions, epithiospecifier protein, and the nature of the glucosinolate side chain. Epithiospecifier protein has recently been independently purified and characterized[7,8] and appears to require ferrous ions indicating the formation of an organometallic (Fe^N - aglycone) intermediate that leads to the formation of a thiirane ring.

CHARACTERIZATION OF APHID MYROSINASE

We have recently reported the first purification of an insect myrosinase and its partial characterization from the cabbage aphid (*Brevicoryne brassicae).*[9] Of all aphids examined for myrosinase,[10] only the crucifer specialists, *B. brassicae* and *Lipaphis erysimi,* possessed activity although some other polyphagous aphids such as *Myzus persicae* can also feed on crucifers.

The native molecular mass of aphid myrosinase, estimated from gel filtration, is approximately 97 kDa, while the molecular mass of the denatured and reduced protein is 53 kDa, estimated from SDS PAGE, and the subunit by MALDI-TOF mass spectrometry 54 kDa ± 500 Da. Thus, aphid myrosinase appears to be a dimeric protein, with identical subunits. The isoelectric points (pI) of aphid myrosinase are 4.90 and 4.95, the latter isoform being the most abundant.

Western blots have shown that the antibody raised to aphid myrosinase (Wye Q) was highly specific to a single band in crude extracts of *B. brassicae* by SDS PAGE analysis.[9] Wye Q did not cross react with proteins (also using Western blotting techniques) from *Sinapis alba* and did not show a reaction to proteins from other *Brassica* pests tested (data not shown). Anti-plant myrosinase antibodies did not cross-react with *B. brassicae* proteins, and anti-aphid myrosinase does not cross react with plant myrosinase. The results of the Western blots are summarized in Table 6.1.

The myrosinase from the turnip aphid, *L. erysimi,* was also partly characterized[9] and shown to cross react with the anti-aphid myrosinase antibody with a single polypeptide of molecular mass 53 kDa ± 2 kDa . Like the cabbage aphid, the turnip aphid myrosinase was not activated by ascorbate in the concentration range 0.1-20 mM (data not shown). The apparent Kms of the aphid myrosinase were 0.613 and 0.915 mM, respectively, for 2-propenylglucosinolate and benzyl glucosinolate, indicating that the enzyme has a greater affinity for 2-propenylglucosinolate. This compares to values of 0.25-0.40 mM for the ascorbate activated plant myrosinase isoforms from *S. alba* and *Brassica napus.*[11] Unlike the plant myrosinase, the aphid enzyme does not appear to be a glycoprotein and is not activated by ascorbic acid. The reactions of both anti-plant and aphid myrosinase antibodies suggest that there are no common epitopes with the plant enzyme.

Table 6.1: Summary of the results of Western blots with anti-plant-myrosinase antibodies and the anti-aphid myrosinase antibody. Wye Q was raised against aphid myrosinase. Wye E, D, and DCJ were all raised against plant myrosinases. + indicates a positive reaction, - indicates a negative reaction. * indicates that this combination was not tested.

Organism	**Antibody Used**			
- Pests	**Wye Q**	**Wye E**	**Wye D**	**DCJ**
Brevicoryne brassicae	+	-	-	-
Myzus persicae	-	*	*	*
Phedon cochleariae	-	+	-	+
Peris rapae	-	+	+	+
Peris brassicae	-	+	+	-
- Plant				
Sinapis alba	-	*	*	*

APHID MYROSINASE GENE

By using aphid myrosinase purified previously, the cDNA sequence of aphid myrosinase was obtained.[12] Five peptides were sequenced and used to design degenerate PCR primers. The full cDNA sequence was obtained using both degenerate and specific primers with Rapid Amplification of cDNA Ends. The GenBank accession number for this sequence is AAL25999.

Aphid myrosinase has significant sequence similarity to plant myrosinases (average 35% protein sequence identity) and other members of glycosyl hydrolase family 1 (GHF1), sharing all six 'Blocks' motifs,[13] and the ProSiteGHF1 motif. Aphid myrosinase is most similar to insect β glucosidases (from the mosquito, *Anopheles gambiae (47%)*, the fruit fly, *Drosophila melanogaster (45%)*, and the cockroach, *Leucophaea maderae(48%)* among others). Aphid myrosinase is also similar to various bacterial β glucosidases (*Bacillus halodurans (37%), Clostridium acetobutylicum (35%), Thermoanaerobacter tengcongensis (35%),* and 6 phospho-β-glucosidase from *Escherichia coli* (29%) among others).

MECHANISTIC DIFFERENCES BETWEEN PLANT AND APHID MYROSINASES

We have previously developed a protein homology model based on the sequence of aphid myrosinase and templates from plant myrosinase and cyanogenic β-glucosidase.[12] These enzymes all adopt the $(\beta/\alpha)_8$-barrel structure typical of this

enzyme family.[14,15] They are globular proteins about 50 Å in diameter with a cleft into the core of the enzyme where the active site is located (Fig. 6.2). The only large structural difference in aphid myrosinase from the plant enzymes is the addition of a loop consisting of residues 270 to 280. The loop was impossible to model accurately but appears to be located on the surface of the protein, folding into two anti-parallel β-sheets.

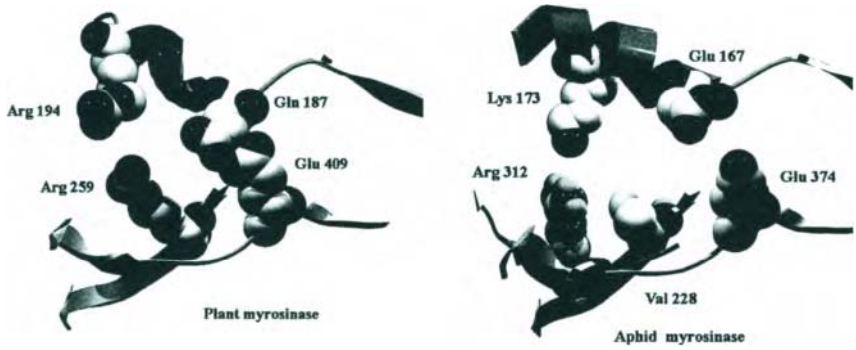

Figure 6.2: Active sites of plant and aphid myrosinases.

The residues acting as proton donor and nucleophile in the hydrolysis of glucosinolates by aphid myrosinase have been identified as Glu 167 and Glu 374, respectively (Fig. 6.2). The equivalent residues in plant myrosinase are Gln 187 and Glu 409 (Fig. 6.2). A generalized mechanism for the aphid myrosinase is shown in Fig. 6.3 and for the plant myrosinase in Fig. 6.4.

Unlike plant myrosinase, ascorbate does not activate aphid myrosinase,[9] and recently the mechanism of activation in the plant enzyme has been determined by using inhibitors together with X-ray crystallography.[16] It was found that ascorbate binds to a site distinct from the glucose binding site but overlapping with the aglycon binding site. This suggests that activation occurs at the second step of catalysis *i.e.,* hydrolysis of the glycosyl enzyme. A water molecule is placed perfectly for activation by ascorbate and for nucleophilic attack on the bound glucose moiety, and thus, ascorbic acid substitutes for the catalytic base in myrosinase.

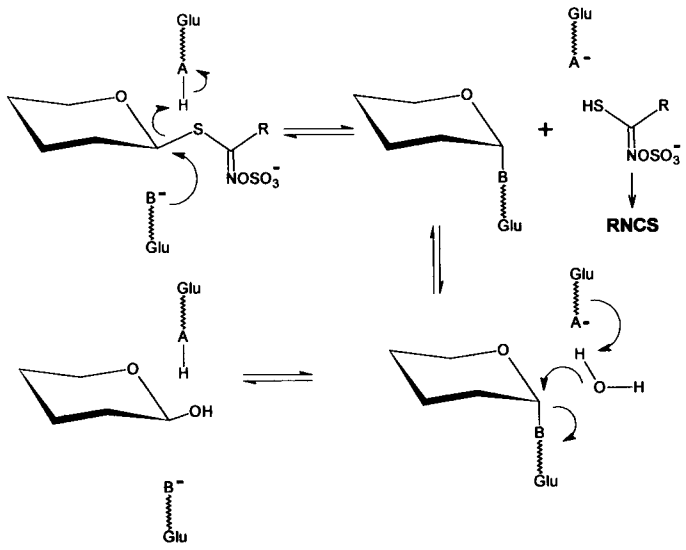

Figure 6.3: A generalized mechanism for aphid myrosinase hydrolysis of glucosinolates.

Recognition of the sulphate group of glucosinolates is probably mediated by residues Arg 194 and Arg 259 within a positive pocket in plant myrosinase. In aphid myrosinase, Lys 173 and Val 228 are similarly positioned, and it is possible that Lys 173, but not Val 228, may play a similar role (Fig. 6.2). In addition, the basic residue Arg 312 is located in the active site and may contribute to recognition of the sulphate group. Amino acid residues that play a part within the active site of plant myrosinase are shown in Fig. 6.5, while potential residues involved in aphid myrosinase are shown in Fig. 6.6.

EVOLUTION

The myrosinase-glucosinolate system occurs in 16 plant families.[17] It is likely that this system evolved from the ancient (and near ubiquitous) cyanogenic glycosides. This hypothesis is supported by the similarity, in sequence and structure, of glucosinolate biosynthetic enzymes and the hydrolytic enzymes (myrosinase or β-glucosidase).[18] The biochemistry of these metabolites also indicates a convincing evolutionary relationship.[19] Furthermore, on the basis of robust phylogenetic trees,

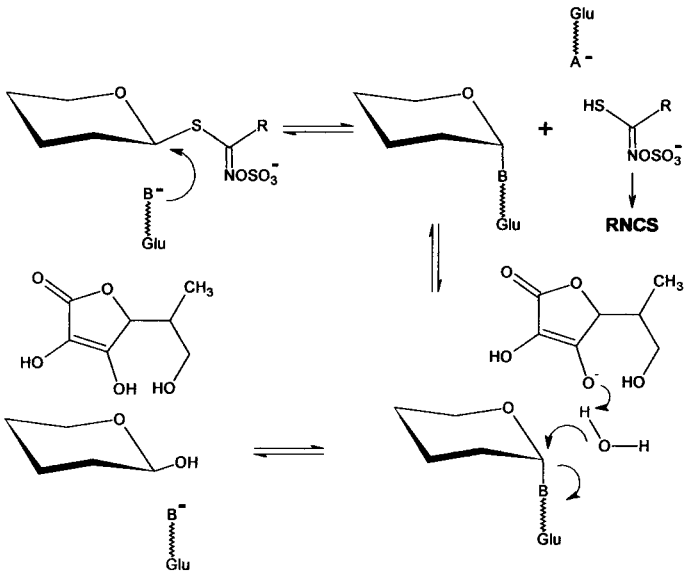

Figure 6.4: A generalized mechanism for plant myrosinase hydrolysis of glucosinolates based on Burmeister et al (transition states are not shown).[16]

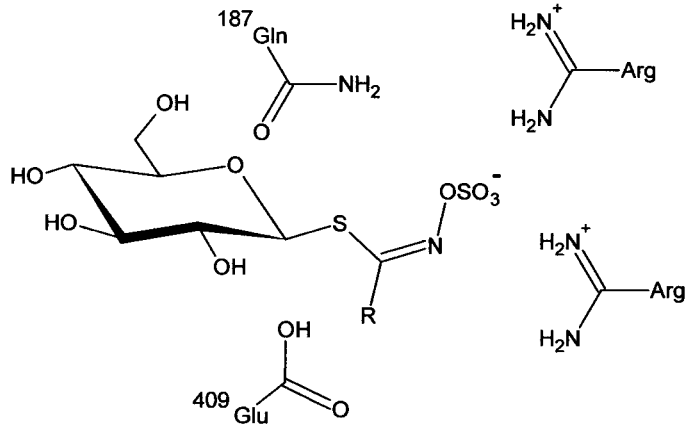

Figure 6.5: Amino acid residues associated with the active site of plant myrosinase.

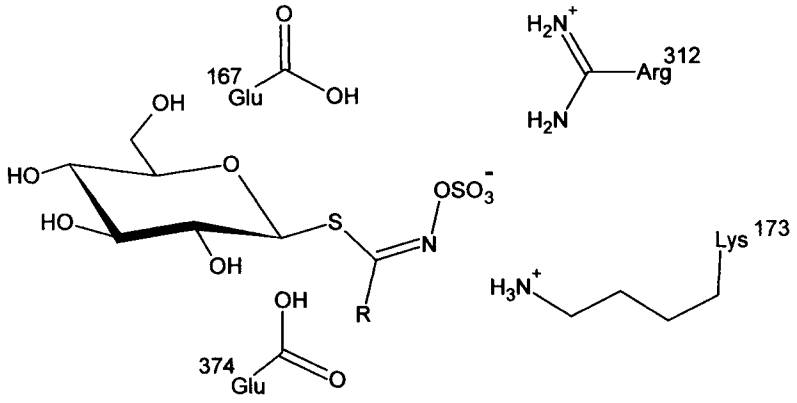

Figure 6.6: Amino acid residues associated with the active site of aphid myrosinase.

Rodman[20] supported an expansion of the order Capparales to include all glucosinolate-producing genera except for the genus *Drypetes* that is placed within the Euphorbiaceae.[21] This suggests that the myrosinase-glucosinolate system has arisen twice in the plant kingdom.

In Jones *et al.*,[12] we presented a phylogenetic tree based on fourteen members of the glycosol hydrolase family 1 (GHF1), including plant and aphid myrosinases. Aphid myrosinase clearly grouped with the only other insect β-glucosidase available at the time (one from *Spodoptera frugiperda*, 46% sequence identity) and mammalian lactase phlorizin hydrolases (known share similarity with both myrosinases and β-glucosidases). Plant myrosinases cluster together; their nearest neighbors are plant β-glucosidases from white clover, *Trifolium repens*, and maize, *Zea mays*. Although myrosinase activity has been reported in bacteria and fungi,[22] to the best of our knowledge there is still no sequence data, despite the many genome sequences currently available.

In conclusion, it seems that myrosinase activity is relatively 'easy' (in evolutionary time) to acquire from β-glucosidases. It will be a fascinating challenge to pin down the exact molecular alterations that are responsible for the transition from β-[*O*]-glucosidase to β-[*S*]-thioglucosidase.

SEQUESTRATION OF GLUCOSINOLATES IN APHIDS

Aphids are phloem sap feeders, and aphids such as *B. brassicae* are specialists, feeding only on plants that contain glucosinolates. Glucosinolates are, therefore, crucial feeding stimulants (phagostimulants), and this was elegantly proved by inducing feeding on broad bean (*Vicia faba,* not a host for the cabbage aphid) with the cut stem dipped in a solution containing 2-propenylglucosinolate.[23] To establish the presence of glucosinolates in the aphid, analysis was carried out on *B. brassicae* and the non cruciferous specialist *M. persicae*. Both aphids were fed on the mustard plant *Brassica nigra,* which contains the glucosinolate 2-propenylglucosinolate, and were analyzed one week later. The concentration of 2-propenylglucosinolate/aphid for duplicate determinations was 102 and 148 ng 2-propenylglucosinolate for *B. brassicae* and 13.2 and 12.2 ng 2-propenylglucosinolate for *M. persicae*. These data strongly suggest that *B. brassicae* has a mechanism for sequestering glucosinolates, while in contrast *M. persicae* accumulates little glucosinolate (which is instead found in the excreted honeydew).[24] This is perhaps not surprising as *M. persicae* does not contain myrosinase, and there is no obvious advantage in sequestering glucosinolates. The actual location of glucosinolates in the aphid is not known at this stage, although circulation in the haemolymph is a distinct possibility, much as cyanogenic glycosides do in the larvae of *Zygaena trifolii*.[25]

In addition the effects of glucosinolates have been studied at a multitrophic level with three Brassicacae species, the aphids *B. brassica* and *M. persicae*, and the polyphagus ladybird beetle, *Adalia bipunctulata*.[26] In these experiments, plants were chosen on the basis of the abundant glucosinolate (*B. nigra*: propenylglucosinolate, *S. alba*: *p*-hydroxybenzylglucosinolate) and *B. napus,* which has a relatively low glucosinolate content. The authors were able to demonstrate a relationship between concentrations of types of glucosinolate sequested in *B. brassicae* and ladybird larval mortality. Interestingly, they found that *B. brassicae* sequested the expected *p*-hydroxybenzylglucosinolate from *S. alba,* but on hydrolysis, crushed aphids appeared to give benzylisothiocyanate. This discovery is quite remarkable as it would appear that not only does aphid myrosinase act as thioglucohydrolase but in addition has the capacity to remove a hydroxyl group from the aromatic ring of *p*-hydroxybenzylglucosinolate.

This work was followed up by studies with purified aphid myrosinase[27] and several glucosinolate substrates. It would appear that not only does aphid myrosinase have the capacity to remove a hydroxyl group from *p*-hydroxybenzylglucosinolate but can also carry out a stereospecific dehydroxylation of (*S*)-2-hydroxybut-3-enylglucosinolate to 3-butenylisothiocyanate rather than formation of the 5-vinyloxazolidinone-2-thione, while (*R*)-2-hydroxybut-3-enylglucosinolate gives rise to 5-vinyloxazolidinone-2-thione (Fig. 6.7). Clearly, the plausibility of this discovery requires revisiting.

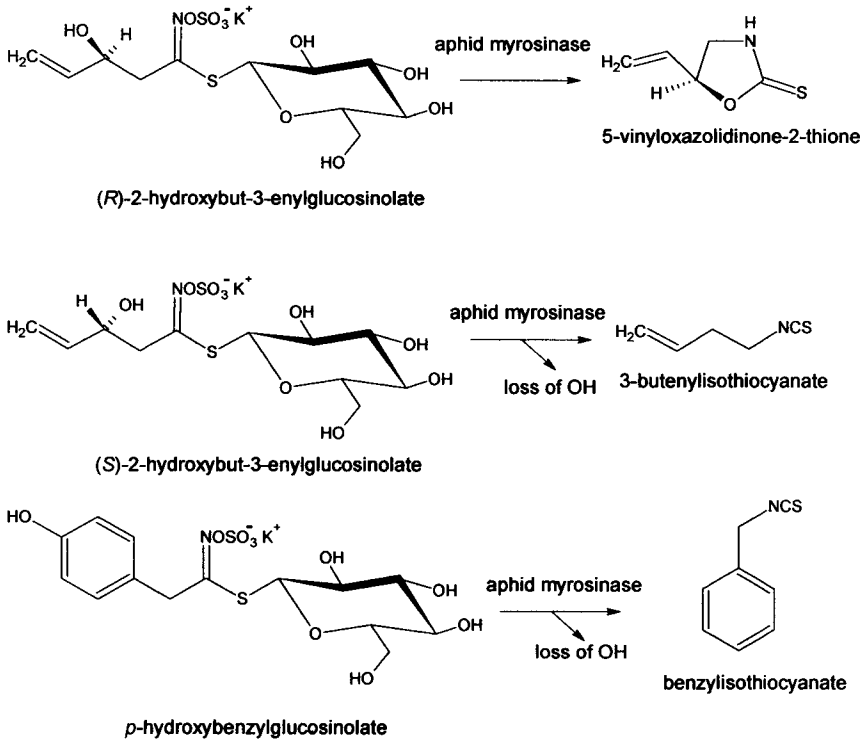

Figure 6.7: Hydrolysis products from 2-hydroxybut-3-enylglucosinolates and *p*-hydroxybenzylglucosinolate with *Brevicoryne brassicae* myrosinase based on Francis *et al.*[22]

SPATIAL ORGANIZATION OF MYROSINASE WITHIN *BREVICORYNE BRASSICAE* AND *LIPAPHIS ERYSIMI*

The localization of aphid myrosinase has been recently reported for the two Brassica specialists *B. brassicae* and *L. erysimmi*.[1] To explore the localization of myrosinase in the aphid, monospecific antibodies (rabbit) were raised to the protein,[9] and immunolocalization was carried out by using both light and transmission electron microscopy.[1] Light microscopy of a transverse section, taken through the abdomen of *B. brassicae* and following immunostaining, showed myrosinase to be present in muscle tissue. A longitudinal section of the head, thorax, and abdomen of

B. brassicae tissue fixed with glutaraldehyde was visualized by light microscopy using silver enhanced immunogold localization and showed the presence of myrosinase over the skeletal muscle of the head, thorax, and abdomen. Labelling was restricted to skeletal muscle, while in all sections examined flight muscles remain free of myrosinase.

Greater magnification (TEM) of the sections showed myrosinase to be present as distinct microbodies in both *L. erysimi* (Fig. 6.8) and *B. brassicae* (Fig. 6.9). In *L. erysimi,* the microbodies were present as crystalline like structures (Figs. 6.8A,C; hexagonal like structures). These microbodies were intensely labelled within the sarcoplasm surrounding the muscles of the thorax and head but were absent from the abdomen and in nymphs (data not shown). Occasionally a microbody was observed within the muscle as an inclusion. Conventionally fixed, osmicated tissue shows dense microbodies (Fig. 6.8D) which appear to be globoidal in comparison to the more regular hexagonal structures present in tissue embedded in LR white and post-stained with uranyl acetate and lead citrate. As with *B. brassicae,* myrosinase was not present in the flight muscles of *L. erysimi.* The structure of the myrosinase containing microbodies in *B. brassicae* (Fig. 6.9) were in comparison to *L. erysimi* (Fig. 6.8) less well defined, although there was some indication (Fig. 6.9A) that hexagonal like structures may exist. A control section is shown in Fig. 6.9B, while an osmicated section is shown in Fig. 6.9C and higher magnification in Fig. 6.9D.

Clearly, the myrosinase-glucosinolate system in the two specialist feeders has an important role in the life cycle of the insect. It is most likely that tissue damage to the aphid by a predator would result in the integrity of the crystalline microbodies being lost, resulting in the formation of a substrate-enzyme complex and subsequent hydrolysis, resulting in the release of volatile material. This spatial organization of aphid myrosinase held in the muscle sarcoplasm separate from glucosinolates, which may circulate in the haemolymph, is perhaps not so different from the plant where myrosinase is restricted to myrosin cells (Fig. 6.8B), though recent evidence suggests that glucosinolates are present in specialized cells of cruciferous plants referred to as 'S' cells.[28] However, in the insect, a more complex regulated mechanism of myrosinase-glucosinolate interaction seems likely, particularly since isothiocyanate release may possibly occur where injury or death is not a pre-requisite for communication. In aggregated insects, especially in groups such as aphids, kin selection could be a dominant evolutionary mechanism. It is likely that the myrosinase-glucosinolate system in the specialist cruciferous aphid feeders acts to maintain a minimum population and is probably most important in the early stages of plant colonization, where a warning signal for predator attack is most useful. A possible direct defense role, in terms of the generation of toxic metabolites for the myrosinase-glucosinolate system against entomopathogenic fungi and the parasitic wasp *Diaeretiella rapae* cannot be ruled out. As with plants (see for example[29]),

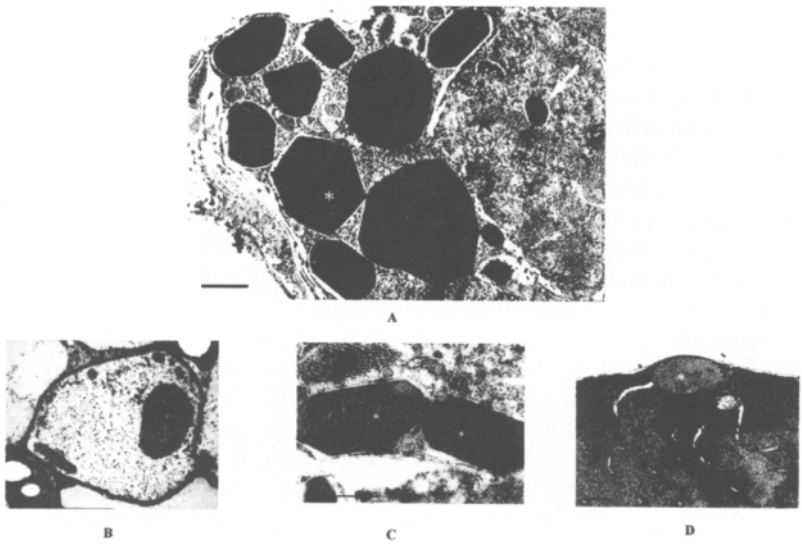

Figure 6.8: Transmission electron microscopy of immunolabelled myrosinase on microbodies contained within peripheral sheath of thorax muscle of *Lipaphis erysimi* and a plant phloem parenchyma myrosinase cell. **A**: crystalline microbodies densely stained with anti-aphid myrosinase antibody. A microbody is also visible included within the muscle fibers (arrow), Bars = μ mm. **B**: transmission electron micrograph of immunolabelled myrosinase on transverse section through proximal region of leaf petiole of *Brassica juncea* at high magnification shows labelling restricted to the myrosin parenchyma cells (anti-plant myrosinase antibody, K089), Bars = 1 μm. **C**: an aphid control section incubated with preimmune serum showing crystalline microbodies, Bars = 1 μm. **D**: conventionally fixed, osmicated tissue showing microbodies within peripheral sheath of the muscle. Section **A** was stained with anti-aphid myrosinase antibody followed by goat anti-rabbit antibody (20 nm colloidal gold). *Black star*, muscle tissue; *black asterisks*, mitochondria; *white asterisks*, dense microbodies. Bars = 1 μm

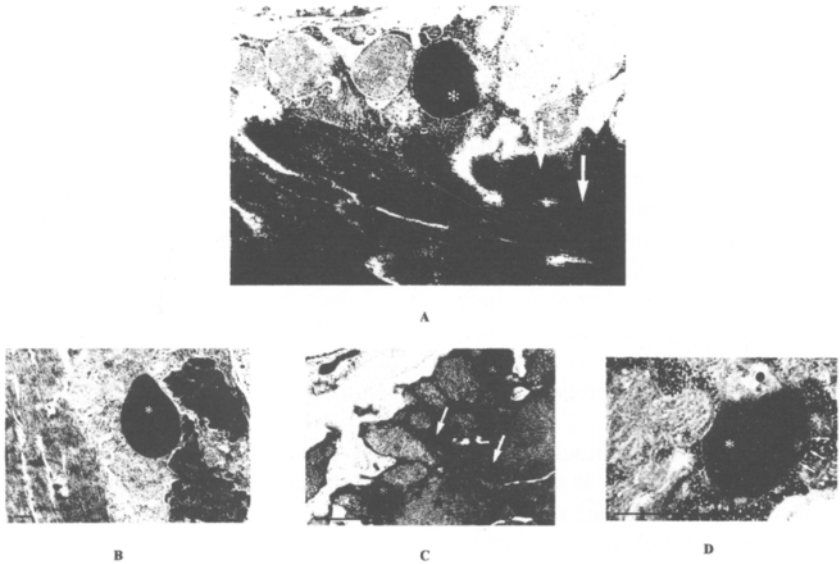

Figure 6.9: Transmission electron microscopy of immunolabelled myrosinase on the thorax muscle of *Brevicoryne brassicae*. **A**: Thorax muscle stained by immunogold labelling showing myrosinase present in a dense microbody within the sarcoplasm of the muscle and on muscle fibres (arrows), Bars = 1 μm. **B**: Control section in pre immune serum, Bars = 1 μm. **C**: Conventionally fixed, osmicated tissue showing dense microbodies in peripheral sheath of muscle. Mitochondria and dense glycogen deposits (arrows) are also visible, Bars = 1 μm. **D**: Increased magnification of osmicated microbody shows a double membrane bounding the microbody. Apart form controls, all sections were stained with anti-aphid myrosinase antibody followed by goat anti-rabbit antibody (20 nm colloidal gold). *Black star*, muscle tissue; *black asterisks*, mitochondria; *white asterisks*, dense microbodies. Bars = 1 μm

these specialist pathogens and predators are probably adapted to the toxic glucosinolate breakdown products, but their effect on non-specialists remains to be determined.

Other insects that utilize glucosides are the Lepidoptera, where it has been shown that β-*O*-glucosidase and hydroxynitrile lyase activity and cyanogenic glucosides are found together in the haemolymph, integument, and organs of the larvae of *Z. trifolii* in varying degrees.[30] Here, how the enzyme and substrate are compartmentalized in the haemolymph is an open question, although some have suggested that pH optima of the linamarase may be a factor in determining regulation of activity. In addition, both calcium and magnesium ions appear to inhibit linamarase in the haemolymph of the insect.[30] Insects have evolved other methods to avoid the toxicity of sequestered secondary metabolites, such as the neotropical butterfly, *Heliconious sara,* which can avoid the harmful effects of the cyanogenic leaves of *Passiflora auriculata* by metabolizing the nitrile group of the cyclopentene ring to a thiol.[31]

Clearly, the way in which the myrosinase-glucosinolate system operates in *B. brassicae* and *L. erysimi* is very different from the cyanogenic glucoside system in other insects, and its organization so far appears to be unique in the insect world, yet draws a direct parallel with the plant system.

SUMMARY

The work described in this review points towards new directions in understanding insect biochemistry and adaptive physiology in insect defense. Aphid myosinase resembles a β-glycosidase both in structure and mechanism and will provide an intriguing protein for structural biologists to study. The location of myrosinase in non-flight skeletal muscle of the aphid signifies an important adaptive evolutionary defense mechanism that mimics the biochemistry of the host plant. The significance and mechanism of the glucosinolate-myrosinase system in the aphid requires a detailed examination not only at a physiological level but also in multitrophic interactions. The cellular mechanisms for the synthesis of aphid myrosinase in a crystalline form, together with the role of the enzyme in multitrophic interactions, represents a fascinating challenge for both cell biologists and entomologists.

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Chapter Seven

MULTIPLE LEVELS OF CONTROL IN THE REGULATION OF ALKALOID BIOSYNTHESIS

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INTRODUCTION

Alkaloids are a diverse group of natural products represented by more than 12,000 structures in about 20% of plant species. These nitrogenous compounds function in the defense of plants against herbivores and pathogens, and are widely exploited as pharmaceuticals, stimulants, narcotics, and poisons due to their potent biological activities. Plant-derived alkaloids in clinical use include the analgesics morphine and codeine, the muscle relaxant (+)-tubocurarine, the antibiotics sanguinarine and berberine, the anticancer agent vinblastine, the antiarrythmic ajmaline, the pupil dilator atropine, and the sedative scopolamine (Fig.7.1). Other important alkaloids of plant origin include the addictive stimulants caffeine and nicotine (Fig.7.1).

Unlike other types of secondary metabolites, each of the many different alkaloid groups has a unique biosynthetic origin. Despite the complexity of understanding alkaloid pathways in general, considerable progress has been achieved on the biosynthesis of several groups due to a number of key technical breakthroughs over the last half century. These include the use of radiolabeled precursors in the 1950s to elucidate pathways, the widespread application of plant cell cultures during the 1970s as a source of biosynthetic enzymes, and the introduction of molecular techniques in the 1990s. Molecular biology has allowed the isolation of numerous genes involved in alkaloid biogenesis, which has facilitated the tissue-specific and subcellular localization of alkaloid biosynthesis, the determination of signal perception events, the elucidation of signal transduction pathways, and the functional analysis of gene promoters. Our current ability to investigate alkaloid biosynthesis from a combined biochemical, molecular, cellular, and physiological perspective has greatly improved our appreciation for the multiple levels of control that function in the regulation of certain pathways. This review will focus on recent advances in our understanding of the regulation of four groups - the benzylisoquinoline (BA), terpenoid indole (TIA), tropane (TA), and purine (PA) alkaloids. All available biosynthetic and regulatory genes are involved in pathways leading to these groups.

ALKALOID BIOSYNTHETIC PATHWAYS

Benzylisoquinoline Alkaloids

BA biosynthesis begins with the conversion of tyrosine to both dopamine and 4-hydroxyphenylacetaldehyde by a lattice of decarboxylations, *ortho*-hydroxylations, and deaminations.[1] The aromatic amino acid decarboxylase (TYDC) that converts tyrosine and dopa to their corresponding amines has been purified, and several

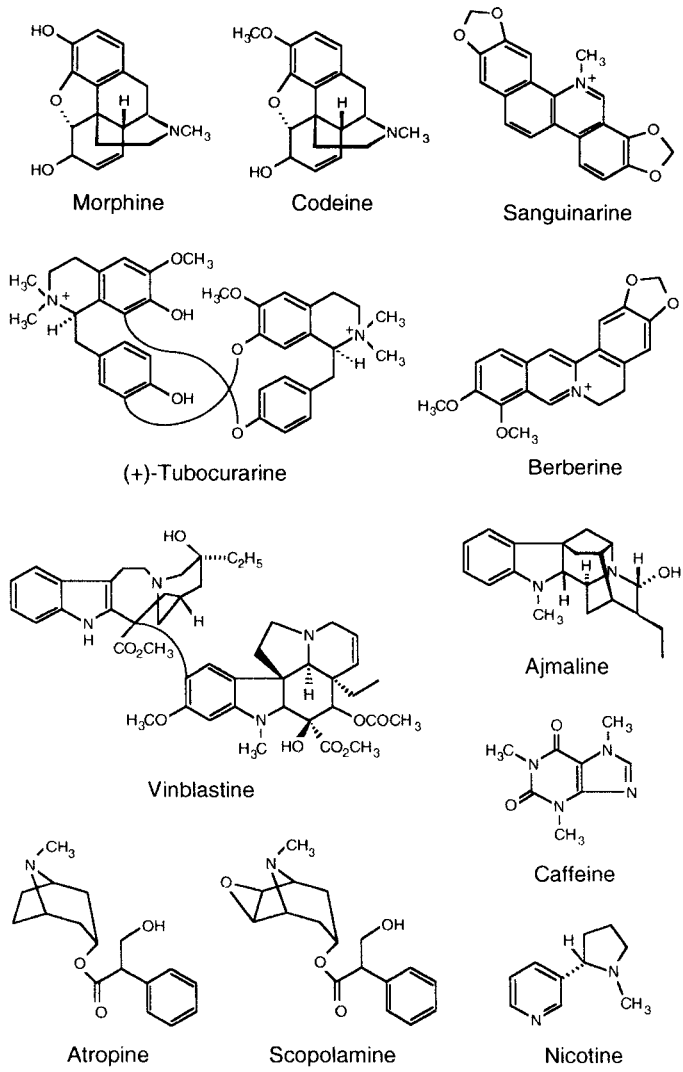


Figure 7.1: Plant-derived alkaloids with important phamacological activities.

cDNAs have been cloned (Fig.7.2).[2,3] A family of ~15 genes, which can be divided into two subgroups based on sequence identity, encodes TYDC in *Papaver somniferum* (opium poppy).[3] The catalytic properties of the various protein isoforms are similar despite the differential developmental and inducible expression of the *TYDC* gene family.[3-5] Dopamine and 4-hydroxyphenylacetaldehyde are condensed by norcoclaurine synthase (NCS) to yield (*S*)-norcoclaurine (Fig.7.2), the central precursor to all BAs in plants.[6,7] NCS will be discussed in more detail later in this chapter.

(*S*)-Norcoclaurine is converted to (*S*)-reticuline by a 6-*O*-methyltransferase (6OMT), a *N*-methyltransferase (CNMT), a P450 hydroxylase (CYP80B1), and a 4'-*O*-methyltransferase (4'OMT) (Fig.7.2).[8-11] 6OMT and 4'OMT were purified from cultured *Coptis japonica* cells, and the corresponding cDNAs isolated and characterized.[8,12] Each enzyme exhibits unique substrate specificity and a different reaction mechanism despite extensive homology. CNMT has also been purified from cultured *C. japonica* cells, and a corresponding cDNA was recently isolated.[13,14] The aromatic ring hydroxylation involved in the conversion of (*S*)-norcoclaurine to (*S*)-reticuline was once thought to proceed via a nonspecific phenol oxidase.[15] However, a P450-dependent monooxygenase (CYP80B1) was shown to have a lower K_m for (*S*)-*N*-methylcoclaurine than the phenolase and is now accepted as the enzyme catalyzing the conversion of (*S*)-*N*-methylcoclaurine to (*S*)-3'-hydroxy-*N*-methylcoclaurine (Fig.7.2).[10]

(*S*)-Reticuline pathway intermediates also serve as precursors to dimeric bisbenzylisoquinoline alkaloids, such as (+)-tubocurarine (Fig.7.1). A phenol-coupling P450-dependent oxidase (CYP80A1) was purified, and the corresponding cDNA was isolated from *Berberis stolonifera*.[16,17] CYP80A1 couples two molecules of (*R*)-*N*-methylcoclaurine or one each of (*R*)- and (*S*)-*N*-methylcoclaurine to form (*R,R*)-guattegaumerine or (*R,S*)-berbamunine, respectively (Fig.7.2). Phenyl ring substitutions, regiospecificity, the number of ether linkages, and monomer stereospecificity add additional diversity to the bisbenzylisoquinoline alkaloids. A cytochrome P450 reductase (CPR) was purified from opium poppy, and corresponding cDNAs isolated from opium poppy and *Eschscholzia californica* (California poppy).[18]

(*S*)-Reticuline is a branch-point intermediate in the biosynthesis of most BAs. Most work has focused on branch pathways leading to the benzophenanthridine (*e.g.*, sanguinarine), protoberberine (*e.g.*, berberine), and morphinan (*e.g.*, morphine and codeine) alkaloids.[19] Most enzymes involved have been isolated, many have been purified, and four corresponding cDNAs have been cloned.[19] The first committed step in benzophenanthridine and protoberberine alkaloid biosynthesis involves the conversion of (*S*)-reticuline to (*S*)-scoulerine by the berberine bridge enzyme (BBE) (Fig.7.2). BBE was purified from *Berberis beaniana*,[20] corresponding cDNAs were cloned from *E. californica* and *B. stolonifera*,[21,22] and *BBE* genes have been isolated from *P. somniferum* and *E. californica*.[23,24]

Benzophenanthridine alkaloid biosynthesis requires the conversion of (*S*)-scoulerine to (*S*)-stylopine by two P450-dependent oxidases, (*S*)-chelanthifoline synthase (CFS) and (*S*)-stylopine synthase (SPS), resulting in the formation of two methylenedioxy groups.[25,26] (*S*)-Stylopine is *N*-methylated by tetrahydroprotoberberine-*cis*-*N*-methyltransferase, which has been isolated from *E. californica* and *Corydalis vaginans* cells,[27] and purified from *Sanguinaria canadensis* cultures.[28] A P450-dependent monooxygenase, (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase (MSH), then catalyzes the formation of protopine.[29] Another P450-dependent enzyme, protopine-6-hydroxylase (PPH), followed by a spontaneous intramolecular rearrangement, converts protopine to dihydrosanguinarine. The oxidation of dihydrosanguinarine to sanguinarine occurs via dihydrobenzophenanthridine oxidase (DBOX),[30] a cytosolic enzyme purified from *S. canadensis* cultures.[31] Two other species-specific enzymes, dihydrochelirubine-12-hydroxylase and 12-hydroxydihydrochelirubine-12-*O*-methyltransferase, catalyze the final two steps in the biosynthesis of macarpine - the most highly oxidized benzylisoquinoline alkaloid found in nature.[32]

In some plants, (*S*)-scoulerine is methylated, rather than oxidized, to yield (*S*)-tetrahydrocolumbamine (Fig.7.2). The reaction is catalyzed by scoulerine-9-*O*-methyltransferase (SOMT),[33] which was purified and the corresponding cDNA isolated from *C. japonica*.[34,35] The next step in protoberberine alkaloid biosynthesis involves the formation of a methylenedioxy bridge.[36] The P450-dependent enzyme canadine synthase (CDS) was detected in members of the genera *Thalictrum* and *Coptis* and shown to catalyze methylenedioxy bridge formation in (*S*)-tetrahydrocolumbamine, but not in the quaternary alkaloid columbamine,[37] showing that berberine biosynthesis cannot proceed via columbamine as once proposed. (*S*)-Canadine, also known as (S)-tetrahydroberberine, is oxidized to berberine either by (*S*)-canadine oxidase (CDO) or (*S*)-tetrahydroprotoberberine oxidase (STOX).[38] These enzymes catalyze the same reaction, but their biochemical properties are distinct. STOX from *Berberis* is a flavinylated protein with a broad substrate range, whereas CDO from *Coptis* and *Thalictrum* contains iron, proceeds via a different mechanism, and prefers (*S*)-canadine as a substrate.

Conversion of (*S*)-reticuline to its (*R*)-epimer is the first committed step in morphinan alkaloid biosynthesis in certain species. 1,2-Dehydroreticuline reductase catalyzes the stereospecific reduction of 1,2-dehydroreticuline to (*R*)-reticuline.[39] Intramolecular carbon-carbon phenol coupling of (*R*)-reticuline by the P450-dependent enzyme salutaridine synthase (STS) results in the formation of salutaridine.[40] The cytosolic enzyme, salutaridine: NADPH 7-oxidoreductase (SOR), found in *Papaver bracteatum* and *P. somniferum,* reduces salutaridine to (7*S*)-salutaridinol.[41] Conversion of (7*S*)-salutaridinol into thebaine requires closure of an oxide bridge between C-4 and C-5 by acetyl coenzyme A:salutaridinol-7-*O*-acetyltransferase (SAT). The enzyme was purified from opium poppy cultures and the corresponding gene recently isolated (Fig.7.2).[42,43] In the last steps of morphine

biosynthesis, codeinone is produced from thebaine and then reduced to codeine, which is finally demethylated to yield morphine. Codeinone reductase (COR), which reduces (-)-codeinone to (-)-codeine, has recently been purified and the corresponding cDNA isolated from opium poppy (Fig.7.2).[44,45]

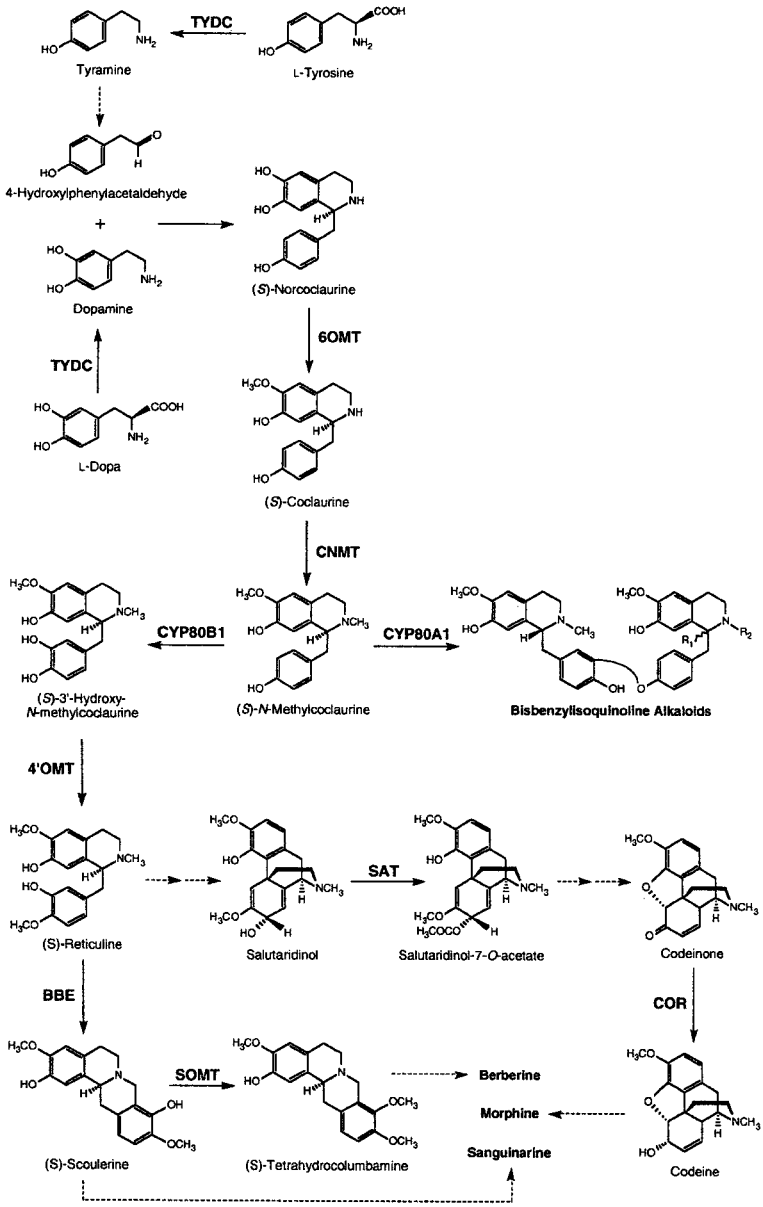
Terpenoid Indole Alkaloids

TIAs consist of an indole moiety provided by tryptamine and a terpenoid component derived from the iridoid glucoside secologanin (Fig.7.3). Tryptophan is converted to tryptamine by tryptophan decarboxylase (TDC), which is encoded by a single gene in *Catharanthus roseus*,[46,47] and by two autonomously regulated genes in *Camptotheca acuminata*.[48] The first committed step in secologanin biosynthesis is the hydroxylation of geraniol to 10-hydroxygeraniol. A novel P450 monooxygenase (CYP76B6) is specific for the C-10 position of geraniol and exhibits similar affinity for nerol, the *cis*-isomer of geraniol. The enzyme was purified and shown to contain FMN and FAD as cofactors,[49] and the corresponding cDNA was recently isolated (Fig.7.3).[50] Conversion of loganin to secologanin represents the last step in the pathway and is catalyzed by another P450-dependent enzyme (CYP72A1) for which the corresponding cDNA has also recently been reported (Fig.7.3).[51,52]

Tryptamine and secologanin condense to form strictosidine, the common precursor to all TIAs, by strictosidine synthase (STR) (Fig.7.3). STR cDNAs have been isolated from *Rauwolfia serpentina* and *C. roseus*.[53-55] Strictosidine is deglucosylated by strictosidine β-D-glucosidase (SGD), which has been purified,[56] and the corresponding cDNA isolated from *C. roseus* cultures (Fig.7.3).[57] Deglucosylated strictosidine is converted via several unstable intermediates into 4,21-dehydrogeissoschizine. Although many TIAs are produced from 4,21-dehydrogeissoschizine, the enzymology of the branch pathways leading to catharanthine and most other alkaloids is poorly understood. However, the final steps of vindoline biosynthesis have been characterized in considerable detail (Fig.7.3). Vindoline is coupled to catharanthine by a non-specific peroxidase to yield vinblastine.[58]

The first of six steps in the conversion of tabersonine to vindoline is catalyzed by tabersonine 16-hydroxylase (T16H) (Fig.7.3), which was characterized as a P450-dependent monooxygenase.[59] A cDNA able to hydroxylate tabersonine at

Figure 7.2: Biosynthetic pathway for benzylisoquinoline alkaloids showing the location of enzymes for which the corresponding cDNAs have been isolated.


TYDC
Tyramine
L-Tyrosine
4-Hydroxylphenylacetaldehyde
Dopamine
(S)-Norcoclaurine
6OMT
TYDC
L-Dopa
(S)-Coclaurine
CNMT
CYP80B1
CYP80A1
(S)-3'-Hydroxy-N-methylcoclaurine
(S)-N-Methylcoclaurine
Bisbenzylisoquinoline Alkaloids
4'OMT
(S)-Reticuline
Salutaridinol
SAT
Salutaridinol-7-O-acetate
Codeinone
BBE
COR
SOMT
(S)-Scoulerine
(S)-Tetrahydrocolumbamine
Berberine
Morphine
Sanguinarine
Codeine

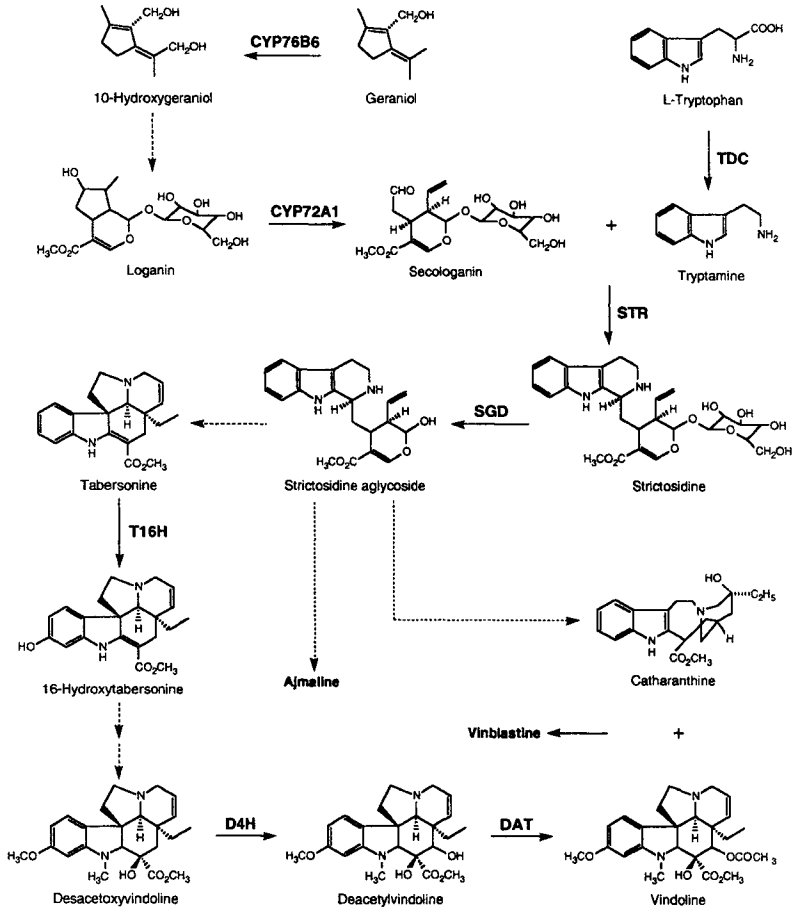


Figure 7.3: Biosynthetic pathway for terpenoid indole alkaloids showing the location of enzymes for which the corresponding cDNAs have been isolated.

the C-16 position was cloned by using a strategy based on the light activation of the enzyme in *C. roseus* cultures.[60] P450 sequences amplified with heme-binding domain-specific primers were hybridized to RNA from induced and non-induced cells. One cDNA (*CYP71D12*), which showed induction kinetics consistent with T16H activity, was capable of converting tabersonine to 16-methoxytabersonine when expressed as a translational fusion with CPR.[60]

The next three steps in vindoline biosynthesis consist of 16-*O*-methylation, hydration of the 2,3-double bond, and *N*-methylation of the indole-ring nitrogen.[61-63] An *O*-methyltransferase has been reported that methylates 16-*O*-demethyl-4-*O*-deacetylvindoline.[64] Initially, two consecutive hydroxylations at the C-3 and C-4 positions were proposed to follow the 16-hydroxylation of tabersonine.[64] However, the isolation of an *N*-methyltransferase (NMT) specific for the indole-ring nitrogen of 16-methoxy-2,3-dihydro-3-hydroxytabersonine showed that the *O*-methylation step precedes *N*-methylation, and that 16-hydroxytabersonine is the natural substrate of the *O*-methyltransferase.[63,65] The enzyme involved in hydrating the 2,3-double bond has not been reported. The final two steps in vindoline biosynthesis are catalyzed by a 2-oxoglutarate-dependent dioxygenase (D4H), which hydroxylates the C-4 position of desacetoxyvindoline,[66,67] and an acetylcoenzyme A-dependent *O*-acetyltransferase (DAT) specific for the 4-OH of deacetylvindoline (Fig.7.3).[68-70] Recently, cDNAs encoding D4H and DAT have been isolated.[71,72] An additional cDNA homologous to DAT was also isolated from *C. roseus* roots and shown to encode the substrate specific enzyme minovincinine-19-hydroxy-*O*-acetyltransferase.[73]

Progress has also been achieved in the isolation and characterization of enzymes involved in ajmaline biosynthesis. Strictosidine could be converted to 10-deoxysarpagine, an intermediate in ajmaline biosynthesis, using protein extracts from *R. serpentina* cultures.[74] The formation of vomilenine, another key intermediate in the pathway, was shown to involve a novel P450-dependent enzyme isolated from *R. serpentina* cells.[75] Vomilenine reductase was recently shown to catalyze the subsequent step in ajmaline biosynthesis.[76] *R. serpentina* cultures normally accumulate the side-product raucaffricine rather than ajmaline. The reutilization of raucaffricine for ajmaline production involves raucaffricine-*O*-β-glucosidase (RG). The cDNA for RG was recently isolated and shown to encode a protein with homology to SGD.[77]

Tropane Alkaloids and Nicotine

The *N*-methyl-Δ^1-pyrrolinium cation is the last common intermediate in both TA and nicotine biosynthesis (Fig.7.4). *N*-Methyl-Δ^1-pyrrolinium cation formation begins with the decarboxylation of ornithine and arginine by ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), respectively. Putrescine is formed

directly from ornithine by ODC, whereas agmatine and *N*-carbamoylputrescine serve as intermediates when putrescine is formed via ADC. An ODC cDNA isolated from *Datura stramonium* showed similarity to ADCs and other ODCs.[78] ADC cDNAs have been isolated from oat and tomato.[79,80] Arginine appears to supply most of the putrescine for alkaloid biosynthesis.[81]

The first committed step in TA and nicotine biosynthesis is catalyzed by putrescine *N*-methyltransferase (PMT) (Fig.7.4).[82] A PMT cDNA isolated from tobacco showed extensive homology to spermidine synthase from mammalian and bacterial sources.[83] *N*-Methylputrescine is oxidatively deaminated to 4-aminobutanal, which undergoes spontaneous cyclization to form the reactive *N*-methyl-Δ^1-pyrrolinium cation. Although the enzymes involved are unknown, the *N*-methyl-Δ^1-pyrrolinium cation is thought to condense either with acetoacetic acid to yield hygrine as a precursor to the tropane ring, or with nicotinic acid to form nicotine.

The first TA-specific intermediate is tropinone, which is located at a branch point in the pathway (Fig.7.4). The 3-keto group of tropinone is reduced to the 3α- and 3β- groups of the stereospecific alkamines tropine and ψ-tropine by two related dehydrogenases, tropinone reductase I (TR-I) and tropinone reductase II, respectively (TR-II) (Fig.7.4). cDNAs encoding the homologous enzymes TR-I and TR-II were isolated from several TA-producing species, but not from tobacco, which accumulates nicotine rather than TA.[84,85] Condensation of tropine and the phenylalanine-derived intermediate tropic acid yields hyoscyamine. Finally, 6β-hydroxylation of the tropane ring followed by removal of the 7β-hydrogen converts hyoscyamine to its epoxide scopolamine (Fig.7.4). Both reactions are catalyzed by hyoscyamine 6β-hydroxylase (H6H), for which a cDNA was isolated from *Hyoscyamus niger*.[86]

Purine Alkaloids

The PA caffeine is produced from xanthosine via three distinct *N*-methylations (Fig.7.5).[87-89] Partially purified enzyme extracts from tea (*Camellia senensis*) and coffee (*Coffea arabica*) were shown to exhibit all three activities, suggesting either that the *N*-methyltransferase steps in caffeine biosynthesis are catalyzed by a single enzyme, or by multiple enzymes with similar properties.[90] However, a specific *N*-methyltransferase purified from coffee was active only toward 7-methylxanthine and theobromine.[91] An *N*-methyltransferase catalyzing the methylation of methylxanthines and designated caffeine synthase (CS) was purified from tea.[92] CS catalyzes two consecutive methylations involved in the conversion of 7-methylxanthine to caffeine, but is inactive toward xanthosine, indicating that the first methylation proceeds via a different enzyme. Heterologous expression of the CS cDNA showed that the enzyme was active toward 7-methylxanthine, paraxanthine,

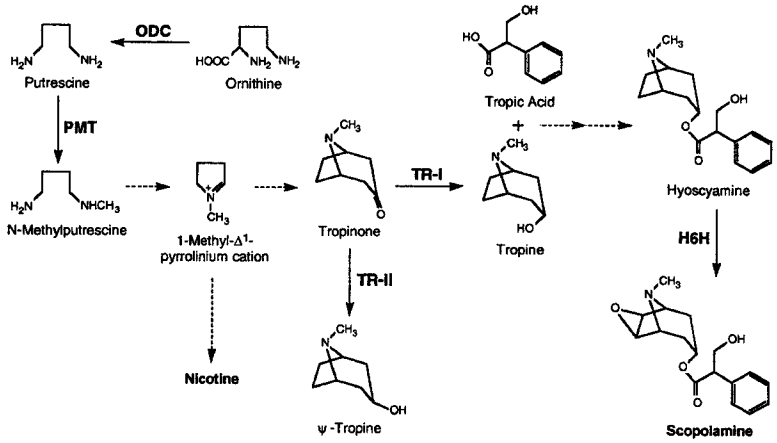


Figure 7.4: Biosynthetic pathway for tropane alkaloids showing the location of enzymes for which the corresponding cDNAs have been isolated.

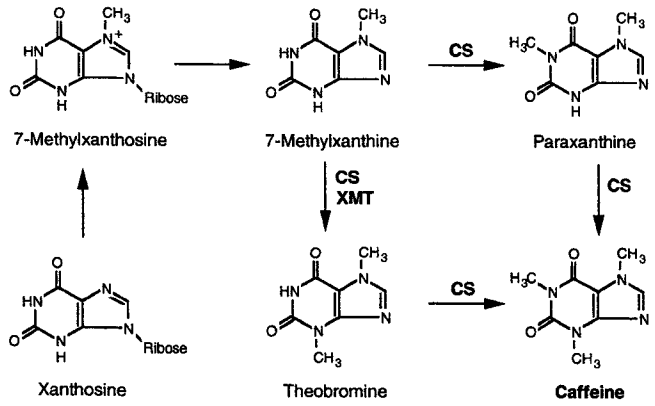


Figure 7.5: Biosynthetic pathway for purine alkaloids showing the location of enzymes for which the corresponding cDNAs have been isolated.

and theobromine (Fig.7.5).[93] However, a cDNA was recently isolated from coffee encoding a methyltransferase (XMT) that catalyzes the conversion of 7-methylxanthine to theobromine (Fig.7.5).[94] XMT did not accept other biosynthetic intermediates, suggesting that caffeine is synthesized via several independent methyltransferases in coffee.

CONTROL MECHANISMS OF ALKALOID BIOSYNTHESIS

Gene Expression and Signal Transduction

Light-Induced Regulation

Several enzymes - TDC, STR, T16H, NMT, D4H, and DAT - involved in vindoline biosynthesis are developmentally controlled in *C. roseus* seedlings, and some are further regulated by light.[59,62,95] Dark-grown *C. roseus* seedlings accumulate tabersonine and smaller amounts of other vindoline pathway intermediates.[61] The transfer of etiolated seedlings to light caused the turnover of tabersonine, and other intermediates, to vindoline, suggesting that some enzymes in the pathway are light-regulated. The light-activation of T16H probably occurs at the transcriptional level.[59,60] NMT and D4H activities also increased when etiolated seedlings were exposed to light.[66,95] The *D4H* gene appears to be under multi-level developmental and light-mediated regulation in *C. roseus* since D4H activity was low in etiolated seedlings despite an abundance of transcripts.[71,96] Exposure of etiolated seedlings to light caused a rapid increase in D4H activity without an increase in transcript levels.[71] Despite the presence of only one *D4H* gene in *C. roseus*, several protein isoforms were detected in etiolated and light-grown seedlings, suggesting that D4H is affected by light at the posttranscriptional level.[96] DAT activity was also found to increase in response to light, but the induction appears to occur only at the transcriptional level.[72,95] Phytochrome was implicated in the activation of vindoline biosynthesis by light,[97] and was shown to control *D4H* gene expression.[96]

Inducible Regulation

Several alkaloid pathways are induced by treatment with fungal elicitors, heavy metal ions, UV radiation, and osmotic shock. Traditionally, work on the inducible regulation of alkaloid biosynthesis has relied on the use of cell cultures treated with fungal elicitors. For example, treatment of *C. roseus* cultures with a fungal elicitor typically increases the accumulation of tryptamine and TIAs, such as catharanthine.[98] Fungal elicitors were shown to induce *TDC*, *STR*, and *SGD* gene expression, suggesting that elicitor-mediated signal transduction pathways are

relatively short and activate pre-existing transcription factors.[55,57] Fungal elicitors also induce jasmonic acid (JA) biosynthesis in *C. roseus* cell cultures (Fig.7.6A).[99] Treatment of *C. roseus* seedlings with methyl jasmonate (MeJA) caused an increase in TDC, STR, D4H, and DAT activities and enhanced vindoline accumulation.[100] TDC and STR transcript levels were also induced in *C. roseus* cultures treated with MeJA or the JA precursor α-linolenic acid.[99] A JA biosynthetic inhibitor, diethyldithiocarbamic acid, blocked elicitor-induced JA fromation, and TDC and STR activation. Moreover, the elicitor-induced biosynthesis of JA and the MeJA-induced expression of *TDC* and *STR* were blocked by the protein kinase inhibitor K-252a, suggesting the involvement of protein phosphorylation in the signal transduction pathway (Fig.7.6A). The JA-mediated induction of TDC and D4H in *C. roseus* seedlings involves transcriptional and post-translational controls.[101] For example, MeJA treatment activated *TDC* expression and increased protein stability, but did not result in higher enzyme activity in light- or dark-grown seedlings. Similarly, exogenous MeJA induced D4H activity and protein levels, but only in light-grown seedlings.

The necessity of a functional octadecanoid pathway for TIA biosynthesis was shown in *C. roseus* cells cultured in an auxin-starved medium.[102] Auxin inhibited TIA accumulation and reduced *TDC* transcription,[47] whereas alkaloid biosynthesis could be induced in an auxin-free medium.[103] Exogenous MeJA restores the ability of cells grown in the presence of auxin to produce alkaloids.[102] In cells cultured in auxin-free media, MeJA or JA treatment causes a further increase in alkaloid accumulation, whereas alkaloid production is reduced in auxin-starved cells treated with octadecanoid pathway inhibitors. These results suggest that JAs are produced in response to auxin depletion and function in coordinating biochemical events that lead to alkaloid biosynthesis (Fig.7.6A). However, despite the role of JAs in linking physiological and environmental signals to alkaloid biosynthesis, JA treatment of etiolated seedlings did not enhance TDC activity, nor did it replace the light requirement for *D4H* expression.[101] In the plant, JAs might function only to modulate events already controlled by other mechanisms.

Leaf damage caused by herbivores also increased JA and nicotine levels in *Nicotiana sylvestris* roots.[104] Exogenous MeJA induced nicotine production in roots, suggesting that JAs function in transferring the damage signal from the shoot to the root. MeJA also caused an increase in ODC, PMT, and *S*-adenosylmethionine synthase (SAMS) transcript levels, and the accumulation of putrescine, *N*-methylputrescine, and nicotine in tobacco cell cultures.[105] However, the induction of *SAMS* and *PMT* gene expression by MeJA was blocked by cycloheximide, whereas *ODC* expression was not, suggesting that multiple control mechanisms are involved in the MeJA-mediated regulation of nicotine biosynthesis. Recently, it was shown that insects could reduce the JA-induced accumulation of nicotine by regulating *PMT* gene expression through the activation of ethylene production.[106]

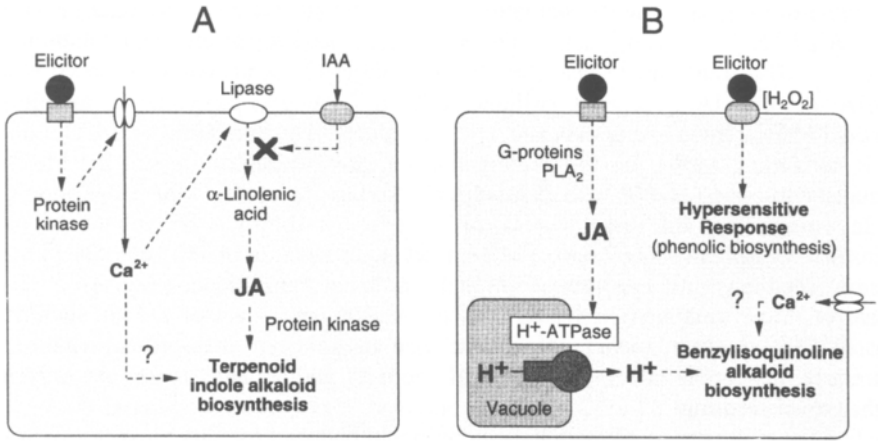


Figure 7.6: Stimulus-response coupling models for the induction of alkaloid biosynthesis in plant cell cultures. A. Terpenoid indole alkaloids. B. Benzylisoquinoline alkaloids.

Cell cultures of several Papaveraceae species accumulate macarpine, sanguinarine, and other benzophenanthridine alkaloids in response to treatment with fungal elicitors.[107-109] In general, membrane-associated enzymes are induced by treatment with elicitor or MeJA.[110] However, some cytosolic enzymes, such as TYDC,[111] NCS,[112] and DBOX were also induced in response to specific treatments.[113] The induction of benzophenanthridine alkaloid biosynthesis in *E. californica* has been shown to occur at elicitor concentrations below the threshold required to stimulate events associated with the hypersensitive response, such as the production of phenolic compounds (Fig.7.6B).[114] Phenolic production could be selectively blocked by catalase at higher elicitor concentrations, suggesting that alkaloid biosynthesis is coupled to a signal transduction pathway that is not mediated by reactive oxygen species involved in activating the classic hypersensitive response. Uncoupled induction mechanisms for phenylalanine ammonia lyase (PAL), TYDC, and downstream enzymes of sanguinarine biosynthesis have also been shown in opium poppy.[111] The elicitor-mediated activation of BA biosynthesis appears to require a transient decrease in cytosolic pH caused by an efflux of protons from the vacuole (Fig.7.6B).[114] Artificial acidification of the cytosol induced BA biosynthesis but not the hypersensitive response, whereas the depletion of vacuolar protons blocked the increase in alkaloid accumulation. Phospholipase A_2 (PLA_2), a G-

protein-controlled redox-dependent plasma membrane protein, might trigger the signal transduction pathway leading to the efflux of vacuolar protons (Fig.7.6B).[115] The role of G-proteins in the induction of BA biosynthesis was also detected in *S. canadensis* cells treated with modulators of GTP-binding proteins and G-protein activators.[116] The induction of BA biosynthesis also appears to depend on an external source of Ca^{2+}, suggesting that Ca^{2+} and perhaps calmodulin participate in the signal transduction pathway (Fig.7.6B).[109]

Promoter Elements and Transcriptional Regulators

Recent studies have identified several promoter elements and transcription factors involved in the regulation of *TDC*, *STR1*, and *CPR*. Three regions involved in basal or elicitor-induced expression were detected from -160 to -37 in the *TDC* promoter.[117] The region from -160 to -99 acted as the main transcriptional enhancer for basal expression, and two separate elicitor-responsive elements were found from -99 to -87, and from -87 to -37. *In vitro* binding of factors in tobacco and *C. roseus* nuclear protein extracts identified two binding activities that interact with multiple *TDC* promoter regions as GT-1 and 3AF1 (Fig.7.7).[118] Mutagenesis of GT-1 binding sites did not affect basal or elicitor-induced expression, but reduced *TDC* promoter activation by UV light.[118]

Coordinate transcript accumulation suggests that *TDC*, *STR*, and *CPR* genes are regulated by common nuclear factors in response to elicitor treatment and UV light.[55,119] Deletion of the *CPR* promoter to -366 eliminated the elicitor-inducible expression observed with a longer promoter.[120] The -632 to -366 region of the *CPR* promoter was also shown to contain GT-1 binding sites (Fig.7.7). The main elicitor-responsive elements of the *STR1* promoter were identified from -339 to -145.[121] Again, GT-1 was shown to bind to this and other regions of the *STR1* promoter (Fig.7.7). Despite the interaction of GT-1 with *TDC, STR,* and *CPR* promoters, the *in vivo* role of this transcription factor in the regulation of gene expression is unknown.

Additional transcriptional regulators must also participate in the expression of *TDC*, *STR1*, *CPR,* and other genes involved in TIA biosynthesis. A G-box motif at -105 binds G-box binding factors (GBFs) *in vitro*, but is not essential for the elicitor-induced expression of *STR1 in vivo*.[121] This G-box element also interacts with tobacco nuclear factors and the G-box-binding factor 3AF1 (Fig.7.7).[122] A G-box tetramer, fused only to a TATA-box, confers seed-specific expression in transgenic tobacco, but requires the enhancer region from the CaMV promoter for expression in leaves. Thus, sequences flanking the G-box motif might be necessary for *STR1* promoter activity in different tissues.[122]

Two other binding factors (CrGBF-1 and CrGBF-2) that bind to the G-box motif in the *STR* promoter were recently isolated (Fig.7.7).[123] A GCC-box element from -100 to -58 of the *STR1* promoter was necessary and sufficient for JA- and

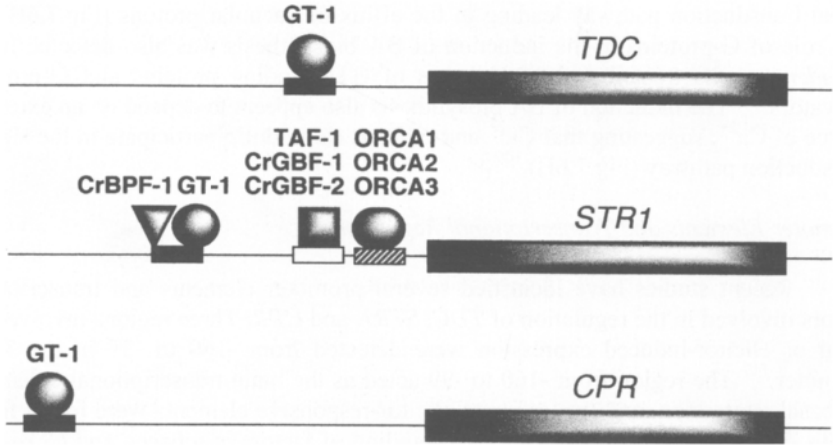


Figure 7.7: The relative location of *cis*-elements and putative transcriptional regulators on the tryptophan decarboxylase (*TDC*), strictosidine synthase (*STR*), and cytochrome P450 reductase (*CPR*) gene promotors from *Catharanthus roseus*. The black box represents elements responsive to elicitor, jasmonate, or UV light. The white box represents a G-box motif, whereas the striped box represents a GCC-box element.

elicitor-responsive expression.[124] Two cDNAs encoding octadecanoid-derivative-responsive *C. roseus* APETALA2-domain (ORCA) proteins that bind the JA- and elicitor-responsive elements of *STR1* were isolated by using a yeast one-hybrid screen (Fig.7.7). The ORCA2 protein *trans*-activates the *STR1* promoter, and expression of the *ORCA2* gene is induced by elicitor and JA treatment of *C. roseus* cell cultures. In contrast, the *ORCA1* gene is constitutively expressed. The closely related *ORCA3* gene was cloned using T-DNA activation tagging and shown to enhance the expression of several TIA biosynthetic genes – *TDC, STR, SGD, CPR,* and *D4H* – when overexpressed in *C. roseus* cultures.[125] ORCA3 was shown to activate gene expression via an interaction with a JA-responsive promoter element.[126] Yeast one-hybrid screening using the STR promoter as bait identified a MYB-like protein in *C. roseus* (CrBPF-1) with homology to a Box P Binding Factor (BPF-1).[127] Unlike the ORCA transcription factors, CrBPF-1 was rapidly activated by elicitor, but not by JA. The induction of CrBPF-1 appears to depend on protein kinase activity and cytosolic calcium concentration (Fig.7.6). These data illustrate

the existence of two distinct transcriptional regulators capable of directing *STR* expression.

Deletion analysis has only begun to reveal the location of regulatory domains in BA biosynthetic gene promoters.[128] The -393 and -287 region of the *TYDC7*, and the -355 and -200 region of the *BBE1* promoters were functionally required in a transient GUS assay system based on the microprojectile bombardment of opium poppy cultures. Time-courses for the induction of TYDC7 and BBE1 mRNAs in wounded opium poppy cells were nearly identical to those for GUS activity in cells bombarded with promoter-*GUS* constructs when the -393 to -287 region of *TYDC7* or the -355 to -200 region of *BBE1* was present. These results suggests that the wound signal caused by the DNA-coated microcarriers induced wound-responsive regulatory elements located from -393 to -287 in *TYDC7*, and from -355 to -200 in *BBE1*. Functional analysis of the *BBE1* promoter from *E. californica* identified the -496 to -455 region as necessary for basal activity.[24] Comparison of this region to the -355 to –200 sequence in opium poppy *BBE1* revealed 55% nucleotide identity within a 40-base pair domain.[128]

Post-Translational Regulation

Enzymatic Controls

Gene regulation represents the most basic level of metabolic control. Although there are few examples in the alkaloid literature, the post-translational regulation of enzymes can also exert considerable influence over the control of metabolic flux. Recent work in our laboratory suggests that enzymatic controls function of the regulation in alkaloid biosynthesis. (*S*)-Norcoclaurine is accepted as the central precursor to all BAs produced in plants.[6,7] However, NCS was first isolated based on its ability to convert dopamine and 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA) to the tetrahydroxylated alkaloid (*S*)-norlaudanosoline.[129] The ability of NCS to accept either 4-HPAA or 3,4-DHPAA contributed to the incorrect conclusion that (*S*)-norlaudanosoline is a common pathway intermediate.[129.130] However, only (*S*)-norcoclaurine has been detected in plants.

We have isolated and purified NCS to homogeneity from *Thalictrum flavum* cultures.[112,131] The purified enzyme displayed native and denatured molecular weights of approximately 28 kDa and 15 kDa, respectively, suggesting that NCS is composed of two subunits. These results are at variance with the native molecular weight of 15.5 kDa previously reported for NCS.[130] Two-dimensional polyacrylamide gel electrophoresis revealed two major and two minor isoforms. The detection of four NCS isoforms is in agreement with the previous characterization of the enzyme.[130] The presence of four isoforms suggests that native NCS exists as either a homodimer consisting of only one isoform, or a heterodimer comprised of

two different isoforms. MALDI-TOF mass spectrometry showed that the major NCS charge isoforms are related. Thus, the native protein complex consists of two similar, if not identical, subunits.

The enzyme displayed hyperbolic saturation kinetics for 4-HPAA (K_m = 335 μM), but sigmoidal saturation kinetics for dopamine (Hill coefficient = 1.8), suggesting cooperative dopamine binding sites on each subunit (Fig.7.8). Cooperative substrate binding suggests that NCS might exert a rate-limiting influence on pathway flux in BA biosynthesis. Invariably, enzymes exhibiting sigmoidal substrate saturation kinetics play a regulatory role in metabolism. *In vitro*, NCS responds to a relatively modest increase in dopamine concentration with a substantial increase in activity, since the binding of dopamine to one subunit increases the affinity for dopamine of the other subunit. However, dopamine was detected at high levels in cultured *P. bracteatum* cells,[132] and is abundant in *P. bracteatum* and *P. somniferum* latex.[133] The dopamine pool was localized within a vacuolar compartment in cultured *P. bracteatum* cells,[132] suggesting that the subcellular trafficking of dopamine represents an additional level of regulation.

Product inhibition kinetics performed at saturating levels of one substrate with norlaudanosoline as the inhibitor showed that NCS follows an iso-ordered bi-uni mechanism with 4-HPAA binding before dopamine (Fig.7.8). These data also imply that 4-HPAA combines with a form of the enzyme different from the alkaloid product since the inhibitor and first substrate do not bind competitively. After the product is released, NCS appears to undergo a conformational change reverting back to a form to which 4-HPAA can bind before another reaction sequence can begin.[131]

NCS shows several features that are similar to STR. Both enzymes catalyze condensation reactions between amine and aldehyde substrates. Four STR isoforms were isolated from *C. roseus* cultures, all of which exhibited cooperative binding kinetics for secologanin.[134] Cooperative substrate binding might have evolved as a parallel mechanism to provide NCS and STR with a regulatory role in their respective pathways. STR and NCS also display similar native molecular weights of 31 and 28 kDa, respectively.[53-55,131] However, STR is composed of a single polypeptide, whereas NCS appears to be a dimer, suggesting that the enzymes are not structurally related.

Metabolic Controls

Many aspects of metabolic control are not readily apparent when isolated enzymes are characterized *in vitro*. The regulatory function of allosteric properties and putative enzyme complexes are best studied *in vivo*. For this purpose, metabolic engineering can be used as a basic research tool to investigate the effect of specific perturbations on the overall flux through an alkaloid pathway. The first application of metabolic engineering to plant alkaloid biosynthesis involved the transformation

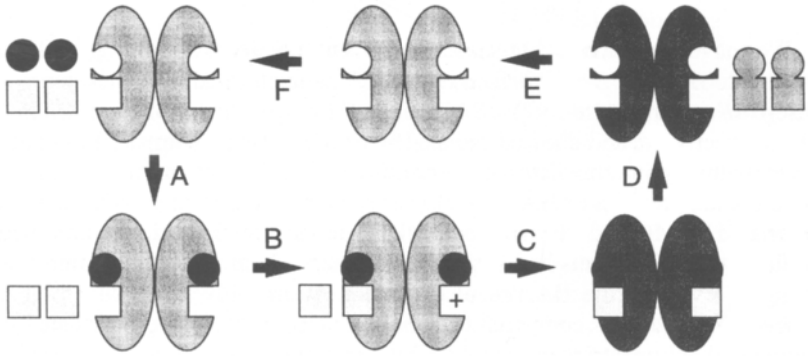


Figure 7.8: Schematic representation of the putative reaction mechanism for norcoclaurine synthase. A. 4-Hydroxyphenyl-acetaldehyde (black circles) binds to the enzyme. B. The binding of dopamine (gray squares) to one subunit of the enzyme increases the dopamine binding affinity of the second subunit. C. A second molecule of dopamine binds to the enzyme. The enzyme undergoes a conformational change (dark gray) during the reaction. D. The product, (*S*)-norcoclaurine, is released. E. The enzyme reverts to a conformation to which 4-HPAA can bind. F. A new reaction sequence begins.

of *Atropa belladonna*, which normally accumulates hyoscyamine, with *H6H* from *Hyoscyamus muticus* resulting in plants that produced high levels of scopolamine.[135] Similarly, expression of *SOMT* in *E. californica* cultures diverted metabolic flux away from benzophenenthridine alkaloids and toward the protoberberine alkaloid columbamine, which does not normally accumulate in this species.[136] In contrast, overexpression of *SOMT* in *C. japonica* cultures resulted in a small increase in berberine accumulation.[136] In fact, most applications of metabolic engineering to alkaloid biosynthesis have involved overexpression of genes encoding key enzymes. For example, *C. roseus* cultures transformed with *STR* accumulated more strictosidine and other TIAs than wild type cultures.[137] In contrast, overexpression of *TDC*, either alone or together with *STR*, did not affect alkaloid accumulation, supporting the role of STR as a rate-limiting step.[137] Similarly, despite strong heterologous *ODC* expression in transgenic *Nicotiana rustica* root cultures, nicotine, putrescine, and *N*-methylputrescine levels increased only slightly, implying that

ODC is not a rate-limiting step in nicotine biosynthesis.[138] Overexpression of oat *ADC* in tobacco increased the accumulation of agmatine, but increased nicotine production was not detected.[139]

We have introduced antisense constructs of genes encoding BBE and CYP80B1 into separate *E. californica* cultures.[140] Transformed cell lines expressing antisense-*BBE* or antisense-*CYP80B1* displayed low levels of BBE or CYP80B1 mRNAs, respectively, and showed reduced BA accumulation compared to controls. Moreover, pathway intermediates were not detected in any of the transformed cell lines, and the suppression of BA biosynthesis reduced the culture growth rate. Two-dimensional ^{1}H-NMR and *in vivo* ^{15}N-NMR spectroscopy showed that transformed cells with reduced BA levels had larger cellular amino acid pools than control cells. Surprisingly, levels of the BA precursor tyrosine were only marginally higher in antisense-suppressed cells compared to controls. Similar results were obtained in *E. californica* root cultures transformed with antisense-*BBE*.[141]

We propose at least three hypotheses to explain the lack of substantial pathway intermediate accumulation in antisense-suppressed cells. The first invokes feedback inhibition by one or more alkaloid intermediates on early biosynthetic enzymes. However, inhibition of enzymes involved in BA biosynthesis by pathway intermediates or end-products has not been demonstrated. The second involves the putative degradation of alkaloid intermediates by an, as yet, uncharacterized mechanism. A third hypothesis raises the possibility that BA biosynthetic enzymes operate as part of a metabolon, or metabolic channel. Although direct interactions between alkaloid biosynthetic enzymes have not been demonstrated, enzyme complexes have been detected in flavonoid biosynthesis.[142,143] The association of an *O*-methyltransferase involved in isoflavonoid biosynthesis with a multienzyme complex might alter its regiospecificity relative to the isolated enzyme by facilitating the direct presentation of substrates into the active site.[143] Removal of components, such as BBE or CYP80B1, from a putative BA metabolon might similarly preclude the accumulation of pathway intermediates due to the lack of coordination among integrated active sites of sequential biosynthetic enzymes. Spatial segregation of BA biosynthesis and end-product sequestration within a cell might also contribute to the lack of (*S*)-*N*-methylcoclaurine or (*S*)-reticuline accumulation. An engineered metabolic block in BA biosynthesis might disrupt the normal metabolic and intracellular transport architecture, thus preventing the accumulation of pathway intermediates or end-products. The reduced growth rate of antisense-suppressed *E. californica* cells might be caused by the impaired metabolic flux of (*S*)-*N*-methylcoclaurine and (*S*)-reticuline, leading to alkaloid intermediate accumulation at marginally cytotoxic levels in an improper subcellular environment.

Cellular Compartmentation and Trafficking

Intercellular Trafficking

Secondary metabolites can accumulate in the same cell and tissue in which they are formed, but intermediates and end-products can also be transported to other locations for further elaboration or accumulation. For example, TAs and nicotine are typically produced near the root apex, but mostly accumulate within leaf cell vacuoles. Even TA biosynthesis itself involves intercellular transport of several pathway intermediates (Fig.7.9A). β-Glucuronidase (GUS) localization in *A. belladonna* roots transformed with a *PMT* promoter-GUS fusion showed that *PMT* expression is restricted to the pericycle.[144] Immunolocalization and *in situ* RNA hybridization also demonstrated the pericycle-specific expression of H6H.[145,146] In contrast, TR-I was immunolocalized to the endodermis and outer root cortex, whereas TR-II was found in the pericycle, endodermis, and outer cortex.[85] The localization of TR-I to a different cell type than PMT and H6H implies that an intermediate between PMT and TR-I moves from the pericycle to the endodermis (Fig.7.9A). Similarly, an intermediate between TR-I and H6H must move back to the pericycle. The occurrence of PMT in the pericycle provides the enzyme with efficient access to putrescine, ornithine, and arginine unloaded from the phloem. In the same way, scopolamine produced in the pericycle can be readily translocated to the leaves via the adjacent xylem.

The translocation of pathway intermediates is also suggested by differential localization of TIA biosynthetic enzymes. *In situ* hybridization and immunolocalization studies have shown that CYP72A1, TDC, and STR are localized to the epidermis of immature leaves, stems, and flower buds.[52,147] In contrast, D4H and DAT are associated with laticifers and idioblasts of shoot organs. Laticifers and idioblasts are distributed throughout the mesophyll in *C. roseus* leaves, and are often several cell layers away from the epidermis. Vindoline biosynthesis involves at least two distinct cell types and requires the intercellular translocation of a pathway intermediate (Fig.7.9B). The differential localization of the early and late steps of vindoline biosynthesis might partially explain why this alkaloid is not produced in dedifferentiated *C. roseus* cell cultures.

The systemic transport of pathway intermediates or end-products might also occur in BA biosynthesis. Several BA biosynthetic enzymes are preferentially active in certain organs. Transcripts encoding various TYDC isoforms were most abundant in roots, or roots and stems.[3] NCS was also most active in roots and stems of opium poppy,[112] whereas CYP80B1 transcripts were most abundant in stems followed by roots, leaves, and floral tissues.[148] STS and SOR, which convert (*R*)-reticuline to salutaridinol, exhibited abundant activity in roots and shoots.[40,41] In contrast, COR

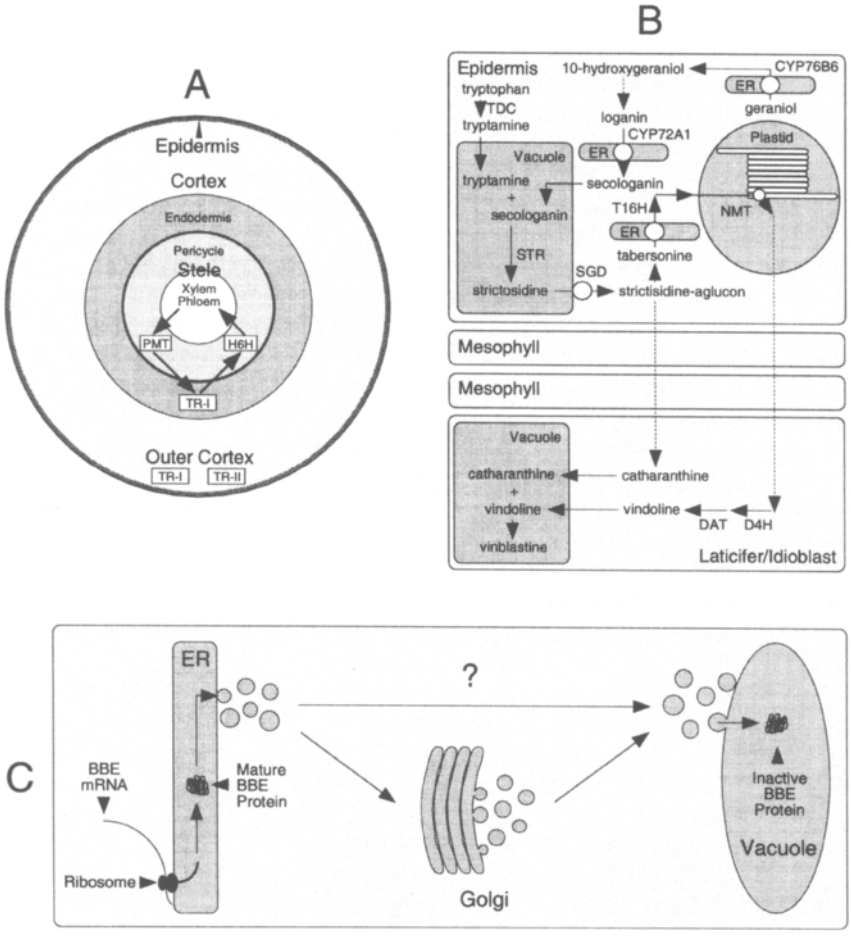


Figure 7.9: Intercellular and subcellular trafficking in alkaloid biosynthesis. A. Tropane alkaloid biosynthesis in *Hyoscyamus muticus*. B. Terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. C. Trafficking of the berberine bridge enzyme in *Papaver somniferum* cell cultures.

was most abundant in shoot organs, but was also found in roots.[45,148] These results suggest that morphine and codeine could be translocated between organs in opium poppy.

In situ hybridization has shown that TYDC transcripts are most abundant in the metaphloem of stems and roots, and are found only at low levels in developing seed capsules.[4] In opium poppy, metaphloem is proximal to the articulated and highly specialized laticifers, which function as a repository for various secondary metabolites in many plants.[149] Alkaloids are stored in the multinucleate laticifer cytoplasm, known as latex. Originally, morphine biosynthesis was thought to occur in laticifers.[149] However, neither of the enzymes STS and SOR could be detected in a fresh latex fraction from opium poppy capsules, suggesting that laticifers are a site of alkaloid accumulation, but not alkaloid biosynthesis.[40,41] Moreover, several enzymes - STS, SOR, SAT, and COR - have been detected in dedifferentiated opium poppy cell cultures despite the absence of laticifers.[40-44] Recent work in our laboratory has shown that CYP80B1, BBE, and COR are localized to a common cell type typically adjacent to, but distinct from laticifers (D. Bird and P. Facchini, unpublished results).

Several different tissue types - epidermis, endodermis, laticifers, idioblasts, pericycle, and cortex – have now been implicated in the biosynthesis and/or accumulation of various alkaloids in plants. Recently, we have localized berberine in the endodermis of *Thalictrum flavum* roots at the onset of secondary growth.[150] Rather than being sloughed off, the endodermis was found to undergo extensive anticlinal division leading to an expanding cellular cylinder that ultimately displaced all external tissues. Endodermal-specific berberine accumulation continued throughout root development, but was extended to include 3 to 4 layers of smaller pericycle cells in the oldest roots near the base of the stem. The cell type-specific accumulation of an antimicrobial alkaloid and the unusual development of the endodermis and pericycle in *T. flavum* roots are consistent with the putative role of berberine in plant defense.

Subcellular Localization of Biosynthetic Enzymes

Many alkaloid biosynthetic enzymes have been localized to subcellular compartments other than the cytosol. Enzyme compartmentation sequesters toxic alkaloids and pathway intermediates away from sensitive areas of the cell. The subcellular trafficking of biosynthetic intermediates might also create an important level of metabolic regulation as suggested for the availability of dopamine to NCS.[131] Understanding the subcellular compartmentation of alkaloid pathways will show whether enzyme characteristics observed *in vitro*, such as the sigmoidal substrate binding kinetics of NCS, represent *bona fide* regulatory mechanisms *in vivo*.

Enzymes involved in vindoline biosynthesis have been localized to no fewer than five subcellular compartments in at least two distinct cell types (Fig.7.9B). The conversion of tryptophan to tryptamine by TDC occurs in the cytosol.[151,152] Since

STR is localized in the vacuole, tryptamine must be transported across the tonoplast before being coupled to secologanin.[153] CYP76B6 and CYP72A1, which catalyze the first and last steps in the conversion of geraniol to secologanin, are endomembrane-bound P450-dependent enzymes.[50,52] CYP76B6 has also been putatively associated with provacuolar membranes.[154] SGD, which catalyzes the deglucosylation of strictosidine, is potentially bound to the external side of the tonoplast,[152] but *in vivo* localization studies showed that SGD is associated with the endoplasmic reticulum (ER).[57] The P450-dependent enzyme T16H, which catalyzes the 16-hydroxylation of tabersonine, is also associated with the ER.[59] NMT is responsible for the third to last step in vindoline biosynthesis and was found to be associated with thylakoid membranes.[65,151] The last two steps in vindoline biosynthesis, catalyzed by D4H and DAT, occur in the cytosol.[66,151] Vindoline must then be channeled to the vacuole where the non-specific peroxidases that produce dimeric TIAs, such as vinblastine, are localized.[58] It should be noted that it is unclear whether some enzymes, such as T16H and NMT, occur in the epidermis, or in laticifers and idioblasts (Fig.7.9B). Nevertheless, the complex compartmentation of the TIA pathway suggests extensive subcellular trafficking of pathway intermediates. The mechanisms involved in channeling intermediates to specific subcellular compartments are not known.

Several BA biosynthetic enzymes also occur in subcellular locations other than the cytosol. Of the five non-cytosolic enzymes involved in the conversion of (*S*)-reticuline to dihydrosanguinarine, four - BBE, CFS, SPS, and MSH - were localized to a membrane fraction with a unique density (δ = 1.14 g mL^{-1}),[25,26,29,38] while one (PPH) was associated with a membrane fraction of the same density as the ER (δ = 1.11 g mL^{-1}).[155] STS, which is involved in morphine biosynthesis, was also localized to a microsomal fraction with a density (δ = 1.14 g mL^{-1}) higher than expected for the ER.[40] With the exception of BBE, all of these non-cytosolic enzymes are P450-dependent and, as such, must be integral proteins of the ER, or ER-derived compartments.[110]

BBE appears to reside as a soluble protein within the lumen of a discrete subcellular compartment.[36,38] A putative N-terminal signal peptide was detected in the deduced amino acid sequence of BBE from *E. californica*.[21] Other soluble enzymes involved in the berberine biosynthesis are also associated with endomembranes of unique density (δ = 1.14 g mL^{-1}), including STOX,[38] CDO,[36] and columbamine *O*-methyltransferase.[156] The association of these enzymes with this particular membrane fraction has led to speculation that distinct *alkaloid synthesizing vesicles* are found in certain cell types.[38] Vesicles (δ = 1.14 g mL^{-1}) containing various alkaloids and biosynthetic enzymes have reportedly been visualized within vacuole-like compartments.[38] We have recently shown that BBE contains a targeting domain comprised of an N-terminal signal peptide and an adjacent vacuolar-sorting determinant.[157] *In vitro* translation of BBE mRNA in the presence of canine pancreatic microsomes produced a glycosylated, proteolysis-resistant protein

confirming the existence of a signal peptide.[157] Transcripts encoding various BBE N-terminal deletions fused to GUS or green fluorescent protein (GFP) were also translated in the presence of canine microsomes, and introduced into cultured opium poppy cells via microprojectile bombardment.[157] The signal peptide was restricted to the first 25 amino acids and shown to initially target BBE to the ER. However, fusion of 50 N-terminal residues from BBE to GFP resulted in the localization of the reporter to the vacuole. GFP was also sorted to the vacuole when fused to a heterologous N-terminal signal peptide followed by BBE amino acids 26 to 50.[157] These data suggest that following the translation of BBE in the ER lumen, the enzyme is transported to the vacuole, possibly as a lumenal component of specific vesicles (Fig.7.9C). BBE is almost certainly inactivated by the acidic conditions in the vacuole, suggesting that alkaloid synthesis occurs before the enzyme crosses the tonoplast membrane. The vacuolar accumulation of sanguinarine in cell cultures suggests that the entire contents of the putative transport vesicles, including BBE and various alkaloid intermediates, are translocated directly from the ER to the vacuole, although the involvement of the *cis*-Golgi network cannot be ruled out (Fig.7.9C).[132,157]

Unique subcellular compartmentation is also present in quinolizidine alkaloid biosynthesis, which occurs in the mesophyll chloroplasts of some legumes.[158] One of the enzymes catalyzing the last two acylations of the pathway in *Lupinus albus* occurs in the cytoplasm, whereas the other resides in the mitochondria.[159] Although the quinolizidine nucleus appears to be synthesized in the chloroplast, subsequent modifications can occur only after alkaloid intermediates are transported to the cytosol and mitochondia. Quinolizidine alkaloids appear to accumulate in vacuoles of epidermal cells where their defensive properties are most effective.

SUMMARY

Alkaloid biosynthetic pathways are under strict regulation in plants. Until now, our limited knowledge of the fundamental mechanisms involved in the control of alkaloid metabolism has severely restricted our ability to harness the vast biotechnological potential of these important secondary pathways. For example, the use of plant cell cultures for the commercial production of pharmaceutical alkaloids has not become a reality despite decades of empirical research. The application of traditional and modern biochemical, molecular, and cellular techniques has revealed important clues about the reasons why *C. roseus* cultures accumulate tabersonine and catharanthine, but not vindoline or vinblastine, and why opium poppy cultures produce sanguinarine, but not codeine or morphine. The inability of dedifferentiated cells to accumulate certain metabolites was interpreted as evidence that the operation of many alkaloid pathways is tightly coupled to the development of specific tissues. Recent studies have shown that alkaloid pathways are regulated at multiple levels,

including cell type-specific gene expression, induction by light and elicitors, enzymatic controls, and other poorly understood metabolic mechanisms. Our ability to exploit the biosynthetic capacity of plants through, for example, metabolic engineering will require a thorough understanding of the mechanisms that allow a cell to produce specific alkaloids. Advances in genomics will provide a more rapid and efficient means to identify new biosynthetic and regulatory genes involved in alkaloid pathways. The apparently unique aspects of alkaloid biosynthesis also provide intriguing targets for plant cell biology research, in general. Novel insights obtained using a combination of traditional and modern technologies, including biochemistry, molecular biology, cell biology, and genetic engineering, highlight the importance of a multifaceted approach in studying the regulation of alkaloid biosynthesis in plants.

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Chapter Eight

BIOCHEMISTRY AND MOLECULAR BIOLOGY OF INDOLE ALKALOID BIOSYNTHESIS: THE IMPLICATION OF RECENT DISCOVERIES

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INTRODUCTION

The monoterpenoid indole alkaloids constitute one of the largest and most complex groups of secondary metabolites produced by plants. They have been shown to occur mainly in the Apocynaceae, the Loganiaceae, and the Rubiaceae plant families, but are also found more sporadically in a few other families. Of the several thousand indole alkaloids that have been characterized, a number have been developed into valuable medicines for the treatment of neurological disorders (reserpine), cancer (vinblastine and vincristine), and as vasodilators (yohimbine).[1-3]

A large amount of structural information is available that describes the variety of indole alkaloids produced in plants. This has recently been followed by significant increases in our knowledge of the biosynthetic pathways that lead to their production and of the genes involved. Several reviews have appeared recently that describe the chemistry, biochemistry, cell and molecular biology of alkaloid biosynthesis.[1-3] This chapter will selectively review recently characterized genes that appear to be responsible for the diversity and complexity of monoterpenoid indole alkaloids produced by plants. A particular focus will be on the reactions leading to the biosynthesis of vindoline (Fig. 8.1) in *Catharanthus roseus*.

CYTOCHROME P450 REACTIONS IN *ARABIDOPSIS* AND *CATHARANTHUS*

Plants contain genes for many cytochrome P450 monooxygenases (P450s) that play key roles in the biosynthesis of the large diversity of natural products found within this kingdom. The *Arabidopsis* genome sequencing effort alone has identified 273 distinct cytochrome P450 genes, although biochemical functions are known for only a few dozen of these. The large size of this gene family in *Arabidopsis* compared to only 57 found in humans and the high substrate specificity of many plant cytochrome P450s provides convincing evidence that the evolution of substrate specific hydroxylation reactions within plants have provided a key ingredient for promoting the large diversity of structures found in nature today. This section will highlight the importance of this class of reaction for the biosynthesis of indole alkaloids.

Geraniol 10 Hydroxylase (G10H)

Geraniol 10 hydroxylase catalyzes the the cytochrome P450 dependent hydroxylation of geraniol at the C-10 position to commit this substrate to the formation of iridoid monoterpenoids (Fig. 8.2). In *Catharanthus roseus*, this intermediate is converted to secologanin for producing the tryptamine containing monoterpenoid indole alkaloids characteristic of this plant.

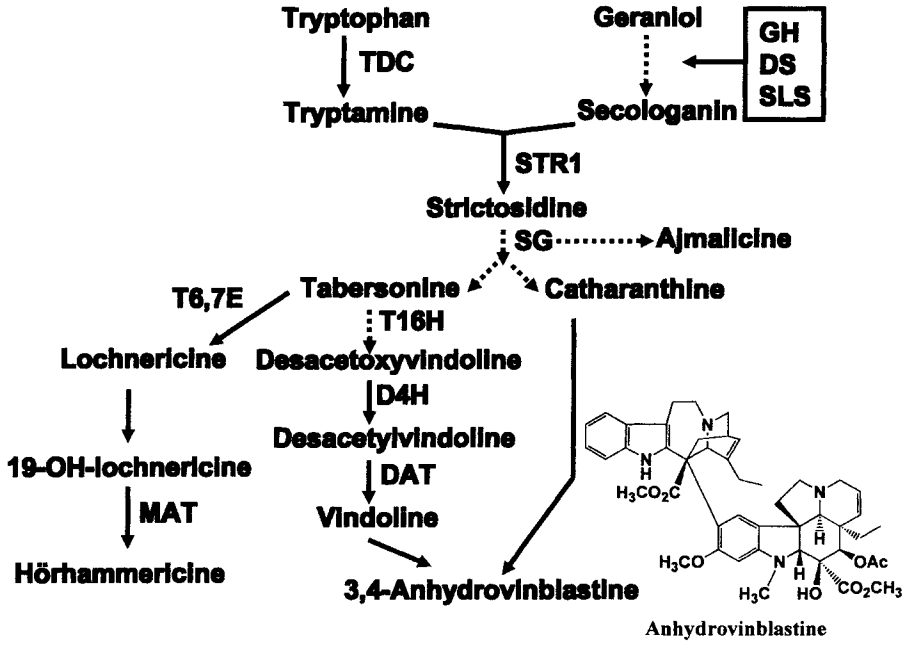


Fig. 8.1: Sequence of reactions and pathways involved in the biosynthesis of indole alkaloids in *Catharanthus roseus*. The dotted lines indicate multiple and/or uncharacterized enzyme steps. Tryptophan decarboxylase (TDC), Geraniol Hydroxylase (GH), Deoxyloganin synthase (DS), Secologanin Synthase (SLS) Strictosidine synthase (STR1), Strictosidine glucosidase (SG), Tabersonine-16-hydroxylase (T16H), Tabersonine 6,7-eposidase (T6,7E), Desacetoxyvindoline-4-hydroxylase (D4H), Deacetyl-vindoline-4-O-acetyltransferase (DAT) and Minovincinine-19-O-acetyltransferase (MAT) represent some of the enzyme steps that have been characterized.

While the majority of biochemical studies have been conducted with the enzyme from *Catharanthus* over the last 3 decades, G10H was first cloned in *Arabidopsis thaliana* by employing a PCR strategy that used degenerate primers derived from the conserved heme-binding domain of P450s. From 16 clones obtained, one of the expressed proteins (CYP76C1) was shown to possess functional

G10H activity.[4] Although the biological role of CYP76C1 in *A. thaliana* is not clear, it is light regulated and its expression is much higher in *Arabidopsis* flowers than in other organs, whereas it is not expressed in roots.[5] This is reminiscent of the flower restricted expression of genes involved in the biosynthesis of the volatile esters in *Clarkia* flowers,[6] and may indicate that *Arabidopsis* produces uncharacterized volatile iridoid monoterpenoids.

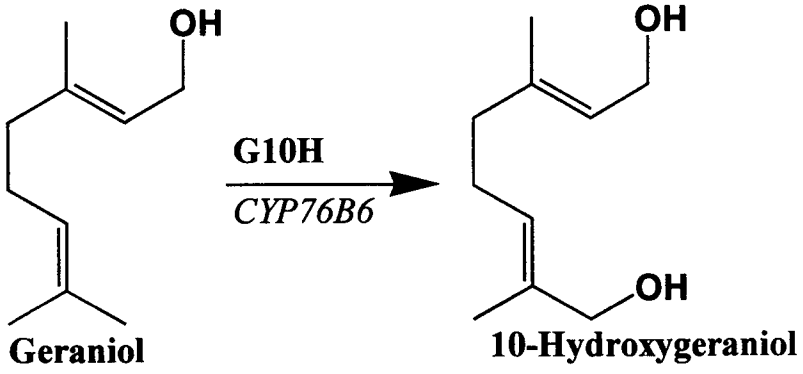


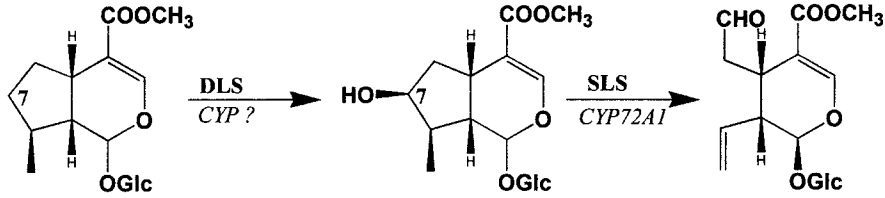
Fig. 8.2: The conversion of geraniol to 10-hydroxygeraniol by geraniol hydroxylase.

Although differential screening[7] and PCR approaches[8,9] have failed to produce G10H clones from *C. roseus*, many cytochrome P450s with unknown function have been isolated. This elusive clone (CYP76B6) was recently isolated,[10] after purifying[11] and partially sequencing homogeneous G10H that provided information for the design of appropriate degenerate primers and cloning. G10H was functionally characterized in both transformed *C roseus* cell cultures and in yeast, showing that geraniol and nerol were converted to their respective 10-hydroxy products.[10] More detailed substrate specificity studies for the clones isolated from *Arabidopsis* and from *Catharanthus* should be considered to define further their range of acceptable substrates, since presence of this hydroxylase in *Arabidopsis* suggests a wider distribution and general function of this reaction type in plants.

Secologanin Synthase

The conversion of loganin to secologanin involves a unique and recently characterized oxidative cleavage of the carbon-carbon bond between C-7 and C-8 on the cyclopentane ring (Fig. 8.3). Given the increased diversity of reactions performed by 2-oxoglutarate dependent dioxygenases and the growing awareness of

the bifunctional nature of many of these enzymes, a plausible mechanism involving dioxygenases would involve the insertion of the iron-oxene into the unactivated C-10 C-H bond of loganin, and coordination of the C7-OH group to the iron atom. This binding would be followed by an oxidative fragmentation reaction leading to the liberation of secologanin and ferrous ion. Efforts to characterize such a dioxygenase in our laboratory have failed (unpublished).



Deoxyloganin **Loganin** **Secologanin**

Fig. 8.3: The conversion of deoxyloganin to secologanin involves two sequential cytochrome P450 dependent monooxygenase reactions.

Recently Yamamoto *et al.* showed that cell suspension cultures of *Lonicera japonica* efficiently convert loganin to secologanin,[12] and clearly established that the reaction is catalyzed by another cytochrome P 450 monooxygenase that requires NADPH and molecular oxygen for activity. Further substrate specificity studies with this enzyme showed that of several closely related substrates tested only loganin served as a substrate for the reaction (Fig. 8.4). Together these results established that secologanin synthase was a highly substrate specific cytochrome P450 monooxygenase. Although the reaction mechanism for this cleavage remains to be established, Akamatsu and Sankawa have proposed that this reaction may proceed via cleavage of the carbon-carbon bond between C-7 and C-8 that causes abstraction of hydrogen radical and produces a carbon radical at C-7 that is converted into an aldehyde group via hydroxylation or desaturation to generate secologanin.[13]

The identification of secologanin synthase as a member of the cytochrome P450 monooxygenases,[12] led to the testing of this enzyme in *Catharanthus roseus* where it was also shown to be associated with microsomes and to belong to the same family of enzymes.[14] This report also showed that the same microsomes contained deoxyloganin 7-hydroxylase activity catalyzed by another possible cytochrome P450 monooxygenase (Fig. 8.3). Additional results also showed that microsomes could convert 7-deoxyloganin in the presence of NADPH into a product that co-migrated on thin layer chromatograms with loganin, and mass spectrometry further confirmed this result.[14]

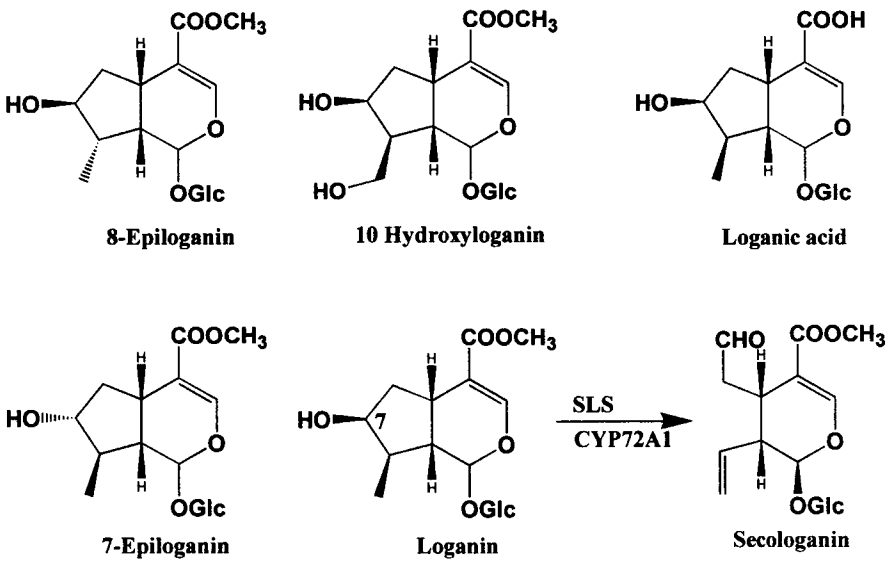


Fig. 8.4: Chemical structures used for substrate specificity studies of secologanin synthase to show that only loganin acts as a substrate for this enzyme.

Although homology cloning techniques have been used to isolate many members of the cytochrome P450s in *Catharanthus*,[7-9] the presence of several hundred of these genes in a typical plant genome, and the variety of the reactions represented, has made it difficult to establish their individual biochemical functions. This strategy has yielded a number of cDNA clones from *Catharanthus roseus* belonging to this family, but several studies have failed to determine biochemical function.[7, 8] When a clone identified as *CYP72A* was expressed as a translational fusion with *C roseus* cytochrome P450 reductase, the resulting protein converted loganin to secologanin.[14] The substrate specificity studies with the CYP72A protein showed it was inactive with geraniol, nerol, flavonoids, cinnamic acid, lauric acid, or 7-deoxyloganin. This result showed that secologanin synthase from *Catharanthus* is highly specific for its substrate as previously shown with the enzyme for *Lonicera japonica* microsomes.[13] The study showed how important functional enzyme assays will be for establishing the biochemical role of the large number of sequenced genes that are available in various databases.

Are There CYPs in Vacuoles and Chloroplasts?

A distinctive feature of *CYP72A1* protein is its lack of a proline rich motif close to the N-terminal membrane anchor that is characteristic of cytochrome P450s. This sequence difference, together with studies in *Catharanthus* cell suspension cultures that show the presence of loganin and secologanin within vacuoles,[15] have been used to speculate[14] that *CYP72A1* may actually be characteristic of a cytochrome P450 targeted to the vacuole (Fig. 8.5), rather than to the endoplasmic reticulum. The speculation is advanced to suggest that this localization would favor the biosynthesis of strictosidine by importing tryptamine from the cytoplasm and conjugating the vacuole located secologanin via a strictosidine synthase that also appears to be located within the vacuoles of *Catharanthus*.[16]

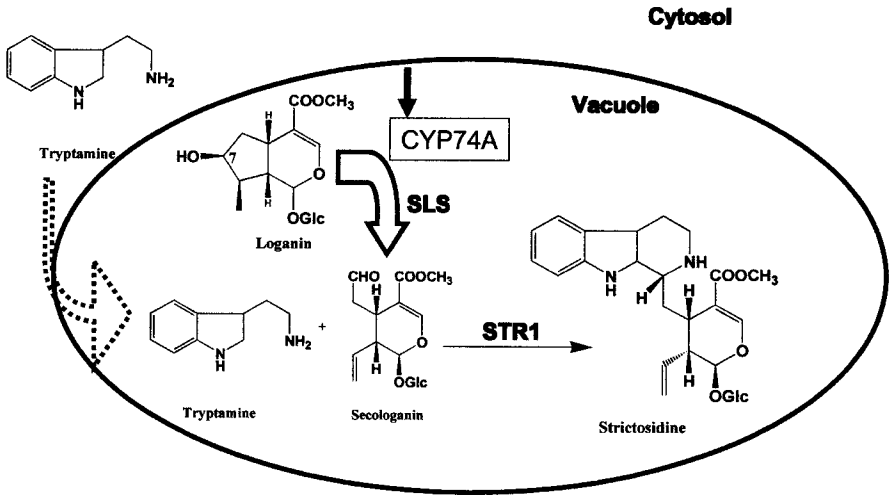


Fig. 8.5: Hypothetical model for the vacuole compartmentation of the reactions leading to secologanin (secologanin synthase, SLS, *CYP72A1*) and strictosidine (strictosidine synthase, STR1) biosynthesis.

There is no clear evidence for this type of localization, since most eukaryotic P450s are anchored on the cytoplasmic face of the endoplasmic reticulum (ER) membrane by a hydrophobic domain in their N-terminus. The association of some cytochrome P450s with the inner mitochondrial membrane in animals,[17] and with the outer envelope membranes of chloroplasts[18] shows that different plant organelles

may also accommodate such reactions. For example, proteins with a high ratio of serine/threonine residues in the amino terminus have been used to define proteins that may be targeted to chloroplasts.[19] Inspection of the 273 cytochrome P450s in *Arabidopsis* have shown that 20 genes contain an appropriate ratio of these amino acid residues in the N-terminus of each protein.[18] *In vitro* chloroplast import assays with *CYP74A* protein (Allene oxide synthase)[20] (Froelich), *CYP86B1* protein (unknown function),[18] and *CYP88A3* protein (entkaurene oxidase)[21] (Helliwell) have also been performed to corroborate their respective associations with chloroplasts. The results show that while *CYP86B1* and *CYP88A3* proteins are exposed to the cytoplasm, they are embedded within the outer chloroplast membrane. In contrast, *CYP74A* protein is inward facing and is embedded in both the inner and outer chloroplast membranes.

The association of cytochrome P450s with organelles other than the ER may have important implications for the biosynthesis of alkaloids. It may help to explain why biochemical localization studies have shown that certain reactions in a pathway occur within the chloroplast. In the case of *Catharanthus* alkaloids, the 3rd to last step in vindoline biosynthesis involves a chloroplast thylakoid associated *N*-methyltransferase.[22] The arguments for participation of chloroplasts in this reaction include the possibility that the previous step involving hydration of the 2,3 double bond might require a chloroplast based oxidation reaction (Fig 8.6). The conclusive identification of specific cytochrome P450 enzymes in chloroplasts suggests that this hypothesis should be tested.

Tabersonine 16 Hydroxylase (T16H)

Tabersonine is converted into vindoline in above ground plant parts via 6 ordered enzymatic reactions that involve hydroxylation, *O*-methylation, an uncharacterized hydration, *N*-methylation, hydroxylation, and *O*-acetylation (Fig. 8.7). The first enzyme converts tabersonine into 16-hydroxytabersonine via a membrane-associated cytochrome P450 monooxygenase whose low activity in etiolated seedlings can be strongly induced by light treatment.[23] Similar studies in cell suspension cultures have also shown that this activity can be strongly induced by light and by transferring the culture to aqueous 8 % sucrose.[24] This result has been used to suggest that cell cultures may be able to accommodate the different cell types required for vindoline biosynthesis.[25]

Putative cytochrome P450 cDNAs were isolated by screening a cDNA library prepared from a light-induced *C roseus* cell suspension culture.[7, 8] Several clones were isolated by using degenerate oligonucleotide primers for conserved sequences around the heme binding site, and a putative *T16H* clone was selected that followed the induction kinetics for the enzyme activity observed upon treatment of cell suspension cultures with light.[24] The biochemical function of this clone was

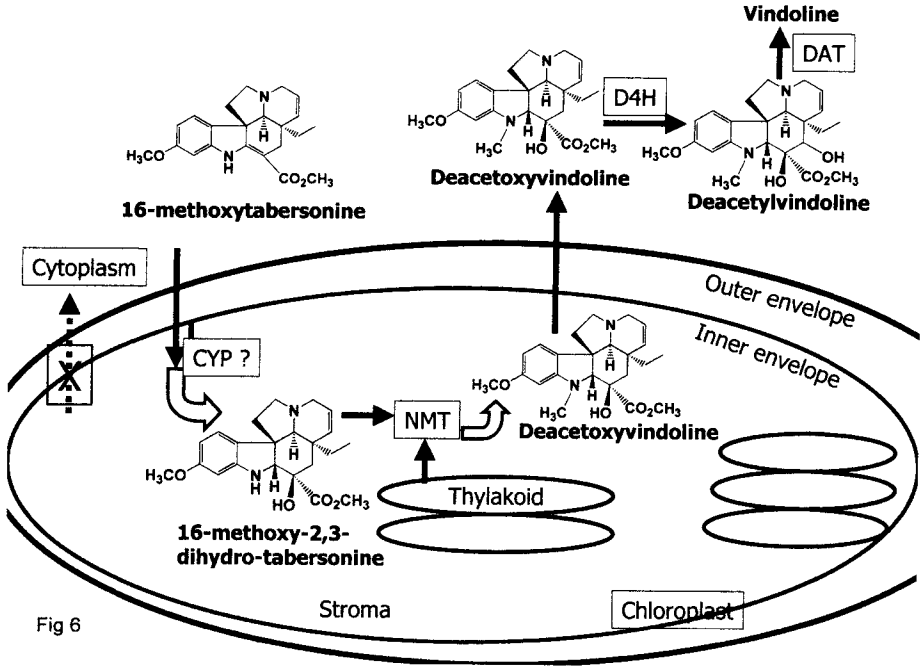


Fig. 8.6: Hypothetical model for the mobilization of 16-methoxytabersonine into the chloroplast in order to accommodate the 4th (hydration of 2,3 double bond) and 3rd (*N*-methylation) to last steps in vindoline biosynthesis.

determined by direct enzyme assay using a soluble recombinant T16H fused to NADPH cytochrome C reductase expressed in bacteria.[24] *T16H* (*CYP71D12*) belongs to the *CYP71D* subfamily of cytochrome P450s that include a number of other genes from different species of plants whose biochemical functions remain to be elucidated. Even though T16H activity is found in cell suspension cultures under certain conditions, it would be of great interest to identify how it is regulated *in vivo* and which of the several cell types in *Catharanthus roseus* plants actually express it, since this would help to identify how much of this pathway actually occurs in roots, leaf epidermis, idioblasts, and/or laticifers.[26]

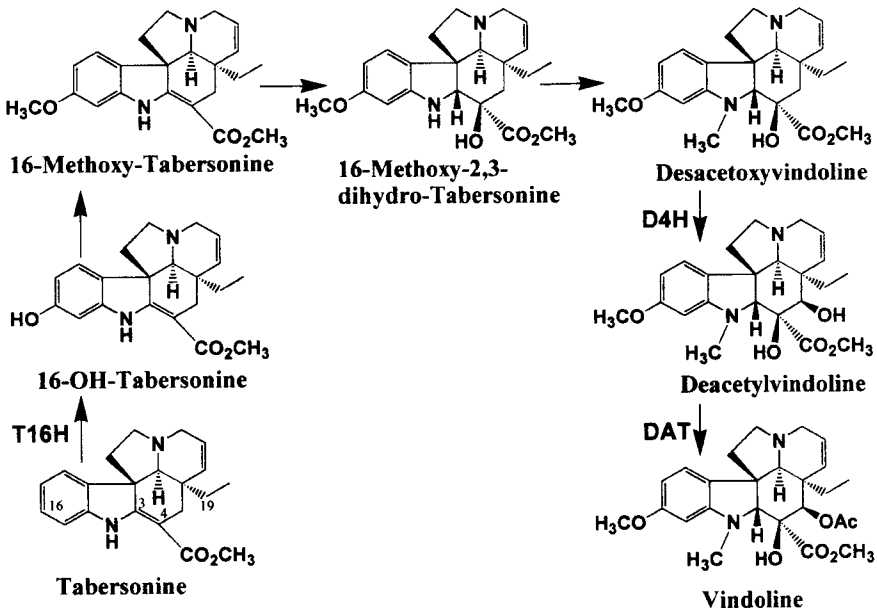


Fig. 8.7: The conversion of tabersonine to vindoline involves an ordered sequence of 6 reactions that include hydroxylation, *O*-methylation, hydroxylation, *N*-methylation, hydroxylation and *O*-acetylation.

Tabersonine 6,7 Epoxidase

Extensive studies to quantitate the production of indole alkaloids in *Catharanthus roseus* hairy root cultures have revealed that they accumulate several compounds including ajmalicine, serpentine, catharanthine, tabersonine, hörhammericine, and lochnericine.[27, 28] The presence of tabersonine in hairy roots has raised speculations that this intermediate in vindoline biosynthesis, together with catharanthine, is transported from this potential site of biosynthesis through the vasculature to the stem and to the leaves where tabersonine is further elaborated into vindoline within laticifers and/or idioblasts.[26] However, oxidized derivatives of tabersonine, such as hörhammericine and lochnericine, are present at 5 to 15 times the levels of tabersonine in hairy roots,[27] and presumably this prevents their transport and/or use for vindoline biosynthesis. In this context, it would be interesting to

determine the rate of biosynthesis of tabersonine to see what proportion of the intermediate is used for the manufacture of these oxidized end products. The pattern of accumulation of tabersonine, lochnericine, and hörhammericine (Fig. 8.8A) [27] suggests that tabersonine is first converted to its epoxide (lochnericine) before further hydroxylation and O-acetylation to form hörhammericine. The data in this report[27] also suggest that the conversion of lochnericine to 19-hydroxylochnericine is probably rate limiting for the production of hörhammericine.

The accumulation of oxidized forms of tabersonine in *Catharanthus* hairy root cultures has prompted studies in our laboratory to characterize the biochemical transformation of tabersonine into hörhammericine via lochnericine, and/or to echitovenine via minovincinine (Fig. 8.8B). Microsomes that were isolated by centrifugation and ultracentrifugation after extraction of 10 to 14 day old hairy roots were assayed for their ability to oxidize ^{14}C-tabersonine (unpublished observations). The results showed that tabersonine could be oxidized into a new product in a reaction that required NADPH and O_2. Since the activity of this microsomal enzyme was inhibited by carbon monoxide and by inhibitors of cytochrome P450s, this reaction also appeared to be part of this family. The oxidized radioactive product was characterized by thin layer chromatography and autoradiography to show that it co-migrated with lochnericine. Analysis of the product by high performance liquid chromatography coupled to UV spectral analysis also confirmed it to be lochnericine, and the enzyme was called tabersonine-6, 7-epoxidase (unpublished observations).

CATHARANTHUS ROOT-AND SHOOT SPECIFIC O-ACETYLTRANSFERASE REACTIONS

The terminal steps in vindoline and 19-O-acetyl hörhammericine/ echitovenine biosynthesis involve separate shoot- and root-specific acetyl coenzyme A-dependent *O*-acetyltransferase genes in Madagascar periwinkle (*Catharanthus roseus* G. Don). These 2 genes showed 63% and 78% nucleic acid and deduced amino acid identity, respectively. The enzymes known as minovincinine-19-*O*-acetyltransferase (MAT)[29] and deacetylvindoline-4-*O*-acetyltransferase (DAT)[30] catalyze the 19-*O*-acetylation of indole alkaloids, such as minovincinine and hörhammericine, and the 4-*O*-acetylation of deacetylvindoline, respectively. The expression of *MAT* helps to explain how roots may accumulate alkaloids like minovincinine, lochnericine, hörhammericine, and echitovenine (Fig. 8.8b).[29] However, kinetic studies showed that the catalytic efficiency of recombinant MAT (rMAT) was poor compared with that of recombinant DAT (rDAT), whose turnover rates for Acetyl-coenzyme A and deacetylvindoline were approximately 240- and 10,000-fold greater than those of rMAT. Northern-blot analyses showed

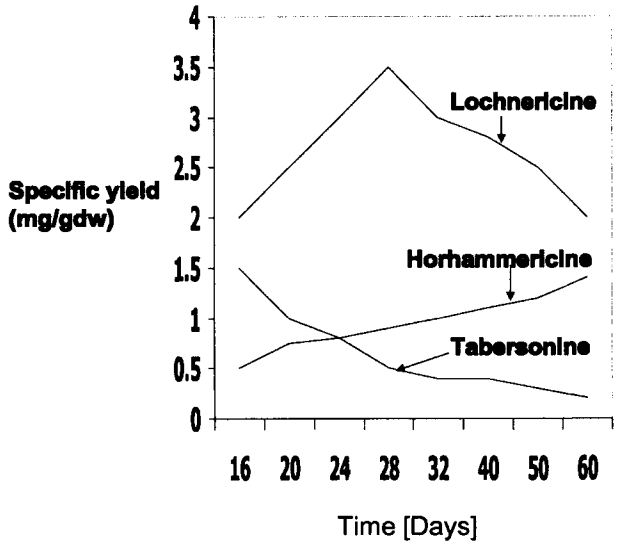


Fig. 8.8A: Accumulation and turnover of tabersonine, lochnericine, and hörhammericine in hairy root cultures of *Catharanthus roseus* over a 60 day period (adapted from Refs. 27 & 28).

that *MAT* is expressed in cortical cells of the root tip, whereas *DAT* is only expressed in specialized idioblast and laticifer cells within light exposed tissues like leaves and stems.[26,30] The coincident expression of trytophan decarboxylase, strictosidine synthase, and *MAT* within root cortical cells suggests that the entire pathway for the biosynthesis of tabersonine and its substituted analogs occurs within these cells. The ability of MAT to catalyze the 4-*O*-acetylation of deacetylvindoline with low efficiency suggests that this enzyme, rather than DAT, is involved in vindoline biosynthesis within transformed cell and root cultures, which accumulate low levels of this alkaloid under certain circumstances.

REACTIONS THAT PRODUCE PRIMARY BACKBONES AND INTERMEDIATES

A key reaction in the formation of alkaloids involves the formation of C-N bonds between amines and aldehydes or ketones. The reaction involves nucleophilic addition followed by the elimination of water to give an imine or Schiff base. The protonated form of this imine is believed to act as an electrophile in a Mannich

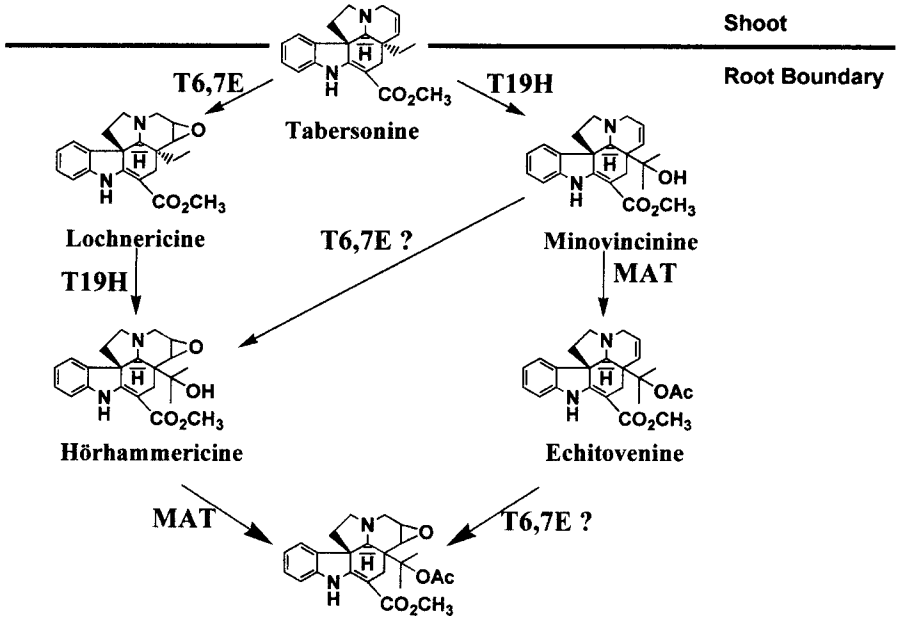


Fig. 8.8B: Reaction grid that converts tabersonine to lochnericine, hörhammericine, minovincinine,echitovenine and 19-acetyl-hörhammericine. The enzymes involved include tabersonine 6, 7-epoxidase (T6, 7E), tabersonine 19-hydroxylase (T19H) and minovincinine-19-O-acetyltransferase (MAT).

reaction, and the nucleophilic addition to the iminium ion might be provided by a carbanion-type nucleophile to yield the C-N bond.

Strictosidine Synthase (STR1)

The enzyme STR1 that was first characterized in *Catharanthus roseus* cell suspension cultures produces the central indole alkaloid intermediate H-3-α-(S)-strictosidine from tryptamine and secologanin (Fig. 8.9). It is well known that strictosidine represents the central intermediate precursor for several thousand indole alkaloids found in Nature. *STR1* was the first gene to be cloned from *R serpentina* that involved a committed step in alkaloid biosynthesis.[31] This was soon followed by the identification and isolation of an *STR* clone from *Catharanthus roseus*[32] whose sequence was 80 % identical to the same gene from *R serpentina*.[31]

The large scale sequencing projects of plant and animal genomes have recently shown that *STR*-like genes exist within both plants and animals that do not produce alkaloids.[33] In fact, humans, rats, fruitfly, and *Caenorhabditis elegans* contain one or 2 *STR*-like genes whose biochemical functions are not known. The insect studies have shown that they produce a cell surface molecule known as hemomucin that is composed of a glycosylated mucin-type repeat domain and an STR1 type domain.[33] Plants such as *Arabidopsis* possess a 13 member family, and strong evidence is accumulating that *STR1* did probably evolve from a common uncharacterized ancestral reaction from which multiple functions appear to have arisen. It is interesting that both hemomucin and STR1 appear to be glycoproteins, although the carbohydrate composition of STR1 has yet to be characterized. Studies on the glycosylation of STR1 should be pursued since this may provide clues about this vacuole associated enzyme activity and the biology associated with alkaloid biosynthesis.

Deacetylipecoside Synthase (DIS)

Alangium lamarckii is a medicinal plant that accumulates tetrahydroisoquinoline monoterpene alkaloids for the production of compounds such as emetine, a constituent of ipecacuantha emetic mixture that is used to for the treatment of drug overdose. Historically, this mixture has also been used to treat amoebic dysentery by South American Indians.

The key step leading to the biosynthesis tetrahydroisoquinoline monoterpene alkaloids is catalyzed by deacetyipecoside synthase (DIS) [34] (Fig 8.9). This enzyme has been purified 570-fold to apparent homogeneity, and has been partially characterized.[34] The purified enzyme is a single 30 Kd polypeptide that catalyzes the condensation of dopamine (Km 0.7 mM) and secologanin (Km 0.9 mM) to form the (R)-epimer of deacetylipecoside. The enzyme shows high specificity for dopamine since neither tryptamine nor tyramine was accepted as substrates.[34] The reaction catalyzed by DIS appears to be the same as that of strictosidine synthase, and it is likely that the *DIS* gene belongs to the same ancestral *STR1* gene family. Research to discover the common primary reaction catalyzed by ancestral *STR1*-like genes will shed light on the evolutionary origins of this gene family.

Norcoclaurine Synthase (NCS)

The stereospecific condensation of dopamine with 4-hydroxyphenylacetaldehyde is catalyzed by NCS to yield *S*-norcoclaurene, the central precursor of thousands of benzylisoquinoline alkaloids, including morphine. This poorly characterized, but key enzyme in benzylisoquinoline alkaloid biosynthesis has recently been purified to homogeneity, and its kinetic properties have been elucidated.[35] NCS occurs as a 28 kDa dimer composed of 15 kDa

subunits, compared to the single 30 kDa monomer that is responsible for strictosidine synthase activity.

Although a Mannich type condensation of an amine and an aldehyde is involved in this reaction, NCS appears to be a different protein from the genes products involved in STR1-type reactions, since it is a dimer containing 15 kDa subunits. Further characterization of this enzyme suggested it belongs to a new, but uncharacterized family of genes (P. Facchini, personal communication).

Strictosidine β-D-Glucosidase (SG)

The versatility of strictosidine as a central intermediate for the biosynthesis of a variety of alkaloids is based on the highly reactive dialdehyde produced by the action of strictosidine β-D-glucosidase. This reactive intermediate is converted by uncharacterized enzymes into the major corynanthe, iboga, and aspidosperma skeletons that are elaborated into the several hundred alkaloids found in *Catharanthus roseus*. Since the biosynthesis of strictosidine appears to occur within plant vacuoles, there has been much speculation, but little is known, about the factors that regulate the accumulation of strictosidine within the vacuole, or which trigger its mobilization for further elaboration. It is well known that glycosides of different natural product classes are located within plant vacuoles.

Early studies to localize strictosidine β-D-glucosidase showed it to be associated with a 100,000 g membrane pellet.[36] Purification of SG to homogeneity showed it occurs as separate isoforms possessing high specificity for strictosidine. The association of this protein with membranes made it difficult to establish its native molecular weight since native polyacrylamide gel electrophoresis indicated the presence of 250, 500, and 630 kDa aggregates, whereas the size of SG when estimated by SDS PAGE was 65 kDa.[36] These results have established that STR and SG occur in different compartments, and it has been suggested that apart from its role as an intermediate in indole alkaloid biosynthesis, strictosidine may be stored in the vacuole for plant defense,[37] since the aglycone has been shown to have antimicrobial activity. In a reaction reminiscent of β-glucosidase induced cyanogenesis,[38] the damage caused by a pathogen would bring strictosidine and SG into contact resulting in the release of the antimicrobial aglycone.

The effort to purify this protein was not useful for cloning *SG*, since the antibodies generated showed poor specificity, and it was difficult to obtain internal amino acid sequences from it. In fact highly conserved consensus sequences from the growing family of plant β-glucosidases were used to make degenerate primers for PCR cloning of a putative 1 kb fragment of *SG* that was used to screen for and to obtain full-length cDNA clones.[39] The cloned *SG* was expressed in yeast where it appeared to form similar large aggregates as those occurring with the native enzyme.

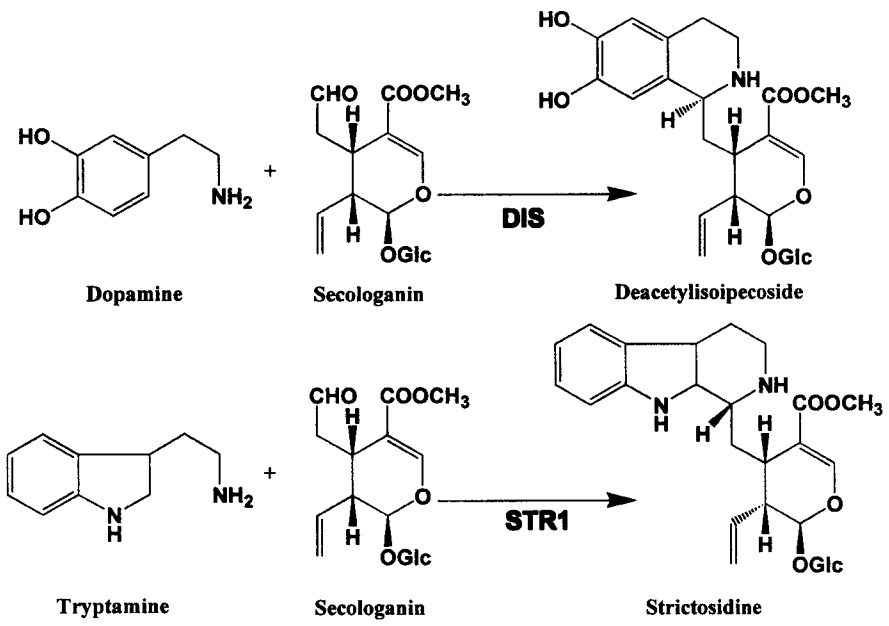


Fig. 8.9: Comparison between the deacetylipecoside synthase (DIS) and strictosidine synthase (STR1) reactions that couple dopamine and tryptamine, respectively, to secologanin.

Crude SG expressing yeast extracts showed high enzyme activity that converted strictosidine into cathenamine.

Although previous studies suggested that SG is highly glycosylated, it appears to contain very little carbohydrate, since its expected Mr of 63 kDa is very close to the size obtained by SDS PAGE.[36, 39] In addition, the enzyme appears to contain a C-terminal sequence KKXKX that is a putative retention signal for type I transmembrane ER proteins. However additional detailed studies must be done to provide evidence for the localization of SG in relation to the site of synthesis of strictosidine.

Raucaffricine β Glucosidase (RG)

Significant efforts to select lines of *R. serpentina* that accumulate high levels of a heart drug known as ajmaline have failed since they tend to accumulate raucaffricine, the glucoside of vomilenine (Fig. 8.10). Cell cultures contain both a

membrane associated glucosyltransferase (SGD) that is responsible for the formation of this glucoside, and raucaffricine β glucosidase (RG) that converts raucaffricine back into vomilenine.[40] Since vomilenine is a direct intermediate in the biosynthesis of ajmaline, it was suggested that cell lines containing high levels of RG might lead to high ajmaline production.[40]

In contrast to SGD, RG appears to be quite soluble, and no problems were reported for its purification from cell suspension cultures of *Rauvolfia serpentina* or to obtain peptide sequence information.[41] In fact, peptide sequencing was used to readily establish that *RG* belongs to the same family of β glucosidases as strictosidine glucosidase.[40, 41] The cDNA for *RG* was recently cloned by RT-PCR using degenerate primers derived from its protein sequence and by screening an *R. serpentina* cDNA library with the cloned PCR product.[41] Although the *RG* clone was not complete, expression of this clone in *E. coli* produced a highly substrate specific protein that accepted raucaffricine, strictosidine, and 5*α*-carboxystrictosidine as substrates, but not a range of others. Although kinetic analysis suggested that Raucaffricine was the preferred substrate for RG, it is unclear whether or not *R. serpentina* also contains an *SG* that is responsible for the cleavage of strictosidine.[41] The isolation of a Rawvolfia *STR* and an *SG* will help to establish if the localized strictosidine biosynthesis observed for *Catharanthus* is more generalized among indole alkaloid producing species.

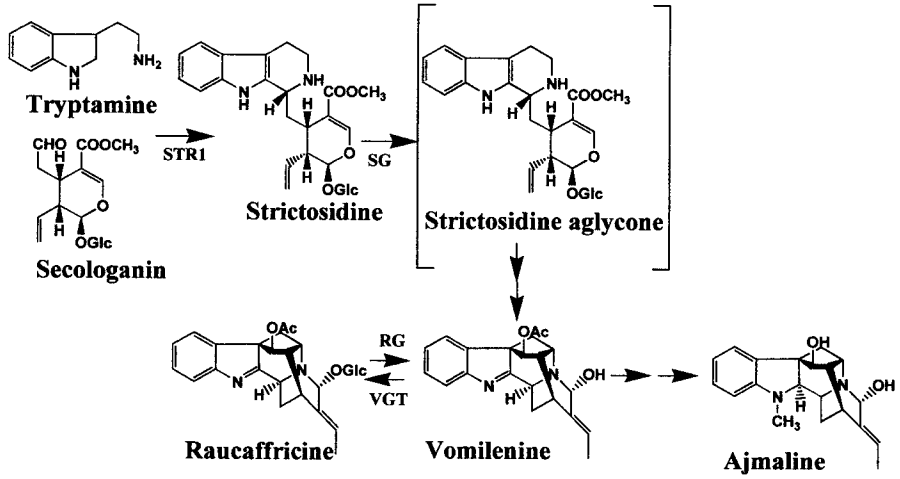


Fig. 8.10: The pathway (adapted from Ref. 40 & 41) for ajmaline biosynthesis in *Rawvolfia serpentina*. The abbreviations represent strictosidine synthase (STR1), strictosidine glucosidase (SG), vomilenine glucosyltransferase (VGT), raucaffricine glucosidase (RG).

SUMMARY AND FUTURE DIRECTIONS

Recent advances in the cell, developmental, and molecular biology of alkaloid biosynthesis have greatly expanded our understanding of their complexity. The studies reviewed here and elsewhere suggest that alkaloids are produced within a small proportion of cells that compose the total organism. Cellular specialization appears to activate: a) the supply of precursors derived from primary metabolic processes; b) specific biosynthetic pathways for converting primary metabolites into particular small molecules; and c) particular sequestering mechanisms, including transport processes that trigger and permit the high level accumulation of end-products. In addition, more than one cell type may be required for the biosynthesis of particular alkaloids, suggesting that alkaloid-specific transport systems have evolved in order to accommodate the process.

The availability of large genomic and proteomic databases together with characterization of the key biochemical functions is now facilitating the complete characterization of alkaloid pathways. The stage is set for large-scale sequencing of cDNA libraries prepared from individual cell-types. The sequencing may confirm that plant cells become natural product biosynthesis factories through targeted biochemical differentiation. Although this strategy has yet to be attempted in plants that produce alkaloids, it has been used with great success to characterize the specialized glandular trichome factories of *Mentha piperita*, where 25% and 7% of the sequenced randomly selected cDNA clones were involved in essential oil synthesis and transport processes, respectively.[42] Similar targeted sequencing of genes expressed in particular cells of alkaloid accumulating plants may yield: a) the complement of genes required to create a particular cell factory; b) tools for targeted expression of novel genes; and c) the regulatory control mechanisms responsible for cellular specialization.

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Chapter Nine

CHEMICAL ECOLOGY OF ALKALOIDS EXEMPLIFIED WITH THE PYRROLIZIDINES

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INTRODUCTION

Within secondary metabolites, alkaloids represent a diverse group with more than 12 000 structures elucidated.[1] According to Pelletier, they are defined as cyclic compounds containing nitrogen in a negative oxidation state that are limited in distribution among living organisms.[2] Though this group encompasses a vast array of unrelated structures, most exhibit substantial biological activities, particularly pharmacological effects on vertebrates.[3] Most alkaloids are easily resorbed, and due to their nitrogenous structure many of them interfere with essential components of the nervous system, acting on presynaptic and postsynaptic receptors, enzymes involved in inactivation of neurotransmitters or in the postsynaptic signal transduction pathway, as well as on specific ion channels. Some alkaloids cause detrimental effects by influencing the function of the cytoskeleton or by interacting unspecifically with DNA or proteins (Table 9.1).[4]

Table 9.1. Examples of toxic plant alkaloids with their respective molecular targets

Alkaloid	Target Site
Nicotine	nicotinic acetylcholine receptor (agonistic)
Curare	nicotinic acetylcholine receptor (antagonistic)
Coniine	acetylcholine receptor (agonistic)
Hyoscyamine (atropine)	muscarinic acetylcholine receptor (antagonistic)
Caffeine	adenosine receptor (antagonistic) phosphodiesterase inhibitor
Morphine	opioid receptor (agonistic)
Physostigmine	acetylcholine esterase inhibitor
Ephedrine	monoamine oxidase inhibitor
Aconitine	activation of Na^+ and K^+ channels
Colchicine	inhibition of microtubule assembly
Taxol	inhibition of microtubule disassembly
Senecionine	alkylation of DNA and proteins after bioactivation

Within flowering plants, approximately 20% of described species contain alkaloids, but in other classes of living organisms, alkaloids have also been detected with restricted distribution among species.[3] As typical secondary compounds, alkaloids have no essential functions for growth and development of the producing organism[5] and are generally constitutively produced in species-specific, genetically defined patterns. There are few examples of inducible alkaloid biosynthesis. Dianthalexin, an alkaloidal phytoalexin, was isolated from elicited tissues of *Dianthus caryophyllus*.[6] An increase in alkaloid accumulation following wounding of plant tissues is well known in tobacco (see below) and has also been reported in *in vitro* systems for indole alkaloids in *Catharanthus roseus*,[7] and for purine alkaloids in tissue cultures of *Coffea arabica* after stress induction.[8]

Alkaloids produced by animals have long been accepted as toxins (*i.e.*, as defense chemicals) against predators.[9] Plant secondary compounds, instead, were regarded as metabolic waste or detoxification products for a long time.[10-12] Although the importance of plant chemicals as protectives against herbivores was emphasized over 120 years ago,[13-15] in the following century scientists lost sight of this functional aspect. In 1959 Fraenkel and in 1964 Ehrlich and Raven, reintroduced the idea of the involvement of these compounds in defense and in the coevolution of plants and insects.[16, 17] This was subsequently supported by understanding the importance of secondary plant metabolites in the ecosystem.[18-22] The view that alkaloids evolved as chemical defenses mainly against herbivores and pathogens and, thus, play an important role in plant fitness, is today well accepted.[3, 9] Due to their biological activity, most alkaloids are toxic to animals, including insects,[23, 24] and their bitter taste is discussed as a general character recorded by animals.[25] Bitterness is important in their role as feeding deterrents. In many cases, the toxic actions are not instantaneous, and delayed detrimental effects may act by weakening the fitness of the herbivore population. The most convincing argument for a defensive role of alkaloids is given by specialized insects that have evolved mechanisms not only to cope with the toxic plant compounds but also to use them for their own defense, sometimes advertising their detrimental load by an aposematic warning coloration.[26]

Alkaloid research began in 1806 with the isolation from opium of the "prinzipium somniferum", morphine, by Friedrich Wilhelm Sertürner. In 1818, W. Meissner coined the term "alkaloid" for the alkalilike compounds of plant origin, and with the synthesis of coniine, the first alkaloid with a proven structure, Ladenburg promoted the era of chemistry in this field. In the middle of the last century, physiologists began tracer experiments to identify precursors and intermediates in the biosynthesis. Mainly by using cell and tissue cultures, biochemists in the 1970s began to identify the specific enzymes involved.[27-30] With the enzymes in hand, cell biologists had the chance to analyze the regulation of the pathways as well as to localize individual enzymes of these pathways.[31-34] Recently, the methods of molecular biology have been introduced to alkaloid research. Genetic tools allow us to understand in more detail the complex regulation and the network of interactions

influencing alkaloid biosynthesis as well as to modify plant secondary product synthesis by gene transfer technology.[35-37] They also allow us to trace the evolutionary origin of alkaloid-mediated plant defenses.[38,39] The statement of Dobzhansky, that nothing in biology makes sense except in the light of evolution,[40] is notably true in the field of chemical ecology. The functional understanding of the ecological basis of the complex system of interactions between the plant and its environment, including more or less adapted herbivores, raises the question of evolutionary origin. Such complex systems are formed by natural selection and can be regarded as the result of "evolutionary molecular modeling".[41] Selection has shaped the structures of alkaloids so that they interact with specific molecular and physiological targets in herbivorous animals. It has optimized their accumulation in sufficient high concentrations in those tissues that allow optimal defense and highest fitness, and it has been the driving force for the development of insect adaptations to these plant defense mechanisms.

For important alkaloid groups like the isoquinolines, the monoterpenoid indoles, or the tropanes, there is only modest evidence concerning their roles in chemical ecology, though they have repeatedly been shown to possess antimicrobial properties or act as efficient toxins or repellents against various groups of herbivores. Among other classes of alkaloids, there are many examples of ecological activities in the literature,[42, 43] but most are not understood mechanistically. This review will present some aspects of the chemical ecology of alkaloids.

ADAPTATION TO ALKALOID-MEDIATED DEFENSE

Defense is never absolute. Despite the high efficacy of alkaloids in the defense of plants that produce them, animals, and mainly insects, have evolved mechanisms of varying degrees of adaptation. Unless there is a shortage of other herbal food, most herbivorous animals avoid alkaloid-containing food, probably being alerted by their bitter taste.[44] In an experiment with sweet and bitter lupines, rabbits clearly prefered the sweet plants, while leaving the bitter plants nearly untouched, demonstrating the greater ecological fitness of the alkaloid plants.[45, 46] In herbivorous insects, powerful olfactory receptors allow them to ascertain the quality of their food plants by detecting the amounts of toxin as well as the nutritional value.[47]

Insects have evolved mechanisms to tolerate plant toxins. Such mechanisms enable them to use a plant as a food source that is avoided by other herbivores, and provides the herbivore with its own ecological niche.[9] For example, insects may prevent accumulation of detrimental alkaloids in the hemolymph by efficient excretion. Larvae of *Spodoptera littoralis* (Lepidoptera, Noctuidae) feed freely on plants containing pyrrolizidine alkaloids that are rapidly excreted and only transiently detectable in the hemolymph. Even pyrrolizidine alkaloids directly injected into the hemolymph are efficiently excreted.[48] The same has been observed

for the migratory grasshopper *Melanoplus sanguinipes*.[49] Inactivation and excretion of toxins by phase I- and phase II-detoxification enzymes is another strategy for coping with plant toxins. An advanced strategy is the development of a target site that is insensitive to a specific toxin. A few vertebrates like guinea pigs and sheep are able to detoxify the pro-toxic tertiary form of pyrrolizidine alkaloids by *N*-oxidation (see below), catalyzed by a microsomal flavin-dependent monooxygenase.[50,51] This explains their resistance to poisoning by pyrrolizidine alkaloids in contrast to cattle, which are unable to detoxify the alkaloids. A cytochrome P450 dependent monooxygenase allows *Drosophila mettleri*, one of four *Drosophila* species endemic to the Sonoran desert in the southwestern United States, to live and breed in soils soaked by rot exudate of the saguaro cactus. This cactus contains toxic isoquinoline alkaloids that accumulate in the soil due to water evaporation [52] and that have been shown to induce a specific P450 monooxygenase.[53] Cytochrome P450 dependent detoxifying enzymes are also assumed to be responsible for the resistance of the tobacco hornworm, *Manduca Sexta*, and other insects to nicotine containing *Nicotiana* species.[54]

A higher level of adaptation and specialization is reached by animals that not only feed on alkaloid defended organisms but in addition have evolved ways to store these compounds for their own defense or use them as precursors for pheromones. The poison-dart frog *Phyllobates terribilis* (Dendrobatidae), for example, sequesters the steroidal alkaloid batrachotoxin from its diet of arthropods, signaling toxicity to potential predators by an intensive warning coloration.[55] An aposematic coloration is also shown by the marine invertebrate *Glossodoris quadricolor*, a nudibranch, that sequesters the toxic alkaloids latrunculin-A and –B after feeding on the sponge *Latrunculia magnifica*.[56] The most intensively investigated examples of this level of adaptation are found within insects that specialize on pyrrolizidine alkaloid containing plants. Probably this is due to the unique feature of this alkaloid group to exist in two interchangeable forms, the non-toxic *N*-oxide and the pre-toxic free base (see below). Arctiid moths, for example, are able to efficiently trap the pyrrolizidine alkaloids as a pre-toxin in their body and in some cases even use these plant derived compounds as precursors for PA-specific insect pheromones signaling their alkaloid load.[48, 57, 58] Some Chrysomelidae leaf beetles accumulate sequestered pyrrolizidine alkaloids in specialized defense glands and later release them in a defense secretion upon predation (see below).[59-61] Even aphids are known to sequester pyrrolizidine alkaloids from the phloem-sap of their food plants. They are transferred to the next trophic level, *i.e.*, ladybirds feeding on the aphids, which themselves accumulate these alkaloids.[62]

An even higher level of specialization is realized by those insects that are attracted by alkaloid containing plants, not for feeding but to sequester them for defense purposes. Adults of some species of the Danaidae and Ihomiinae (butterflies, Lepidoptera) have evolved this special behavior and are attracted by pyrrolizidines and ingest them frequently from dead parts of pyrrolizidine alkaloid containing

plants.[63] This phenomenon is called "pharmacophagy" and shows explicitly that coevolution of plant and insect herbivores not only results in chemical and physiological adaptations but also in a specialized behavior.[64]

PLANT STRATEGY FOR DEFENSE

The production of toxins is only one aspect of plant defense strategy. As a result of the persistent battle of plants and herbivores, many optimized phenotypes have evolved, such as the preferential accumulation of alkaloids in tissues with a pattern that is consistent with predictions of optimal defense theory,[65] *i.e.*, the defense metabolites are allocated preferentially to tissues with a high probability of attack.[66] The inducibility of pathways leading to plant secondary compounds as a strategy to minimize the costs of plant defense is a result of permanent optimization. One of a few examples of inducible alkaloid biosynthesis is the different *Nicotiana* species that exhibit dramatic wound-induced increases of nicotine, nornicotine, or anabasine.[67]

Nicotiana *Plastic Responses Induced By Herbivore Attack*

Nicotiana plants have evolved a battery of plastic responses to herbivore attack. They not only have direct and indirect defenses but also trigger their time of seed germination to escape herbivores, as has been shown for *N. attenuata* (Fig. 9.1).[68] Nicotine induction is one of many defenses induced in tobacco by herbivore attack. Nicotine biosynthesis is activated by the jasmonate cascade, [69] and results in an increase of nicotine concentrations that are 4- to 10-fold above the constitutive level of an uninduced plant.[67, 70] The resulting concentrations are sufficiently high to offer a lethal dose in a single meal for many herbivores and to protect induced tissues even against herbivores that are adapted to nicotine, such as the tobacco hornworm, *Manduca sexta*.[71] About 6% of the total nitrogen content of an induced plant is bound to nicotine, excepting the nitrogen that is temporarily used for biosynthetic enzymes and alkaloid transport. Since this nitrogen is not available for other activities, the induced nicotine production provokes high cost that is reflected in significantly reduced numbers of viable seeds.[68]

Nicotine biosynthesis is localized in the roots of *Nicotiana* plants, and the alkaloids are transported to the shoots in the xylem stream,[70] mainly to young leaves and stems and the reproductive parts of the plant.[72] At first glance, the costly transport mechanisms seem to be a disadvantage, as there is a time lag of 10 hr from time of induction until the increase of nicotine production.[73] The roots, however, as the site of synthesis are well protected against herbivory and continue the production, even when up to 88% of the total leave area is removed.[74] Optimization of the cost-value ratio seems to be the reason for the inducible defense acting as a cost-saving

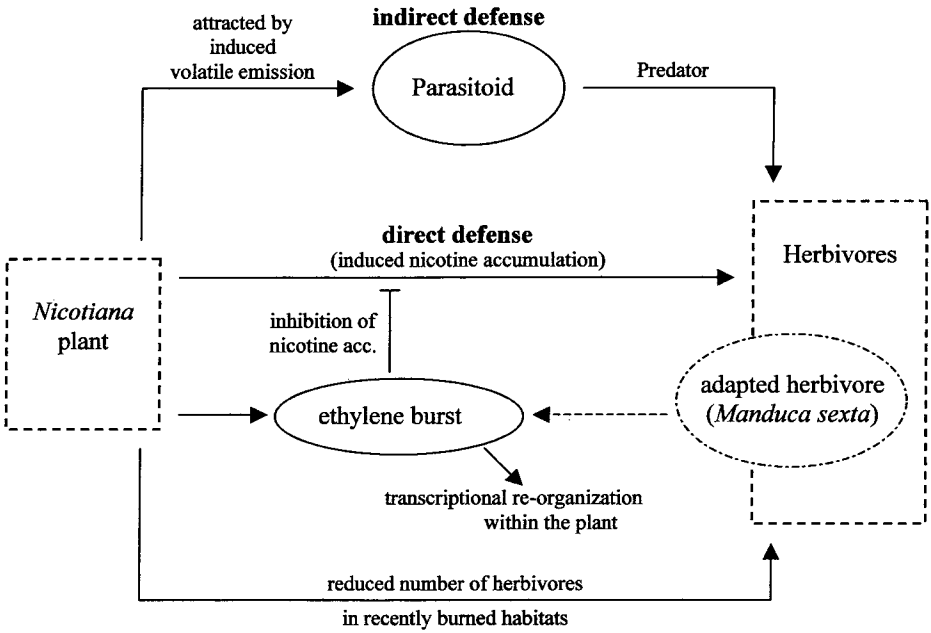

Figure 9.1. Different defense strategies of *Nicotiana* plants that evolved in response to herbivore attack. Besides a specialized germination-behavior that efficiently reduces the over-all number of potential herbivores, *Nicotiana* plants evolved the inducible nicotine synthesis as a direct defense and the inducible emission of volatiles to attract parasitoids of the herbivore as an indirect defense. A specialized defense mechanism is triggered in response to attack by the herbivore *Maduca sexta*, adapted to *Nicotiana* plants. Attack results in an ethylene burst, which down regulates nicotine accumulation and results in a fundamental transcriptional re-organization within the plant.

measure that considers the high costs of alkaloid synthesis.[75] Minimizing the costs for defense was probably also the selective force that resulted in the particular germination behavior. *N. attenuata* germinates in its natural habitat from long-lived seed banks after fires, in an environment that is devoid of most of its herbivores and offers the plants a large supply of soil ammonia that can be used for growth and for its nitrogen-intensive defense.[76, 77] That this germination behavior provides a large fitness benefit is supported by the fact that two independent germination signals trigger the germination process. There is a positive signal from smoke that breaks dormancy of the seeds and a negative signal that acts until germination-inhibiting factors from mature intact vegetation are eliminated by the fire.[78]

Recently, it was discovered that nicotine induction is only one aspect of defense in *Nicotiana*. The jasmonate cascade results in an increase in volatile emission (mono- and sesquiterpenes) as an indirect defense strategy,[79] probably to attract parasitoids of the herbivores, as has been described for many other plants.[80, 81] A broad array of specific responses is initiated if larvae of the tobacco hornworm *Manduca sexta* are feeding on tobacco. A suite of eight fatty acid-amino acid conjugates, identified in the oral secretions and the regurgitants of the larvae, are necessary to induce a specific ethylene burst in *Nicotiana*.[82] This cannot be mimicked by a comparable amount of mechanical damage.[83] The ethylene burst is accompanied by increased levels of jasmonic acid, but it suppresses the induction of two nicotine biosynthetic genes encoding putrescine *N*-methyltransferase, *PMT1* and *PMT2*. As a result, no nicotine is accumulated.[84] While down regulating this direct defense, the emission of volatiles, *i.e.*, the indirect defense, is not affected.[79] Furthermore, *M. sexta* attack of *N. attenuata* results in a fundamental transcriptional re-organization. It has been estimated that more than 500 genes respond to herbivore attack by being up or down regulated.[85]

What is the selective driving force that resulted in this change from a direct to an indirect defense as a response to herbivore attack? One may speculate that larvae of *M. sexta* evolved this mechanism in order to feed on tobacco[66] without the high levels of nicotine that are growth repressive to the larvae.[86] Or did plants evolve this mechanism in response to the nicotine-tolerant herbivores that undermine its cost-intensive direct defense?[66] The herbivore will be more susceptible to parasitoids due to the lower nicotine load, since *M. sexta* is unable to sequester nicotine in its body.[87] Transcriptional re-organization supports the idea that it is the plant that gives a broadside to the herbivore. Another explanation is that the altered wound response is a strategy to suppress the nitrogen-consuming nicotine defense in favor of promoting the plant's growth in a competitive habitat where the plant germinates simultaneously with many conspecifics.[66]

The *Nicotiana* system provides insight into the complex mechanisms evolved by plants in response to herbivore attack.[88] Realizing that a response to an attack is the expression of about 500 genes, one can see how fragmentary our knowledge of these response mechanisms still is.

Origin of Pyrrolizidine Alkaloid Biosynthesis in Plants

In contrast to nicotine formation in *Nicotiana* plants, pyrrolizidine alkaloids (PAs) are constitutively formed and are not inducible by wounding, herbivory, or microbial attack.[89, 90] PAs are ester alkaloids composed of a necine base and a necic acid moiety.[91] In *Senecio* species, the backbone structure of the PAs is exclusively produced in the roots (Fig. 9.2).[92] Dedifferentiated cell suspension cultures established from a number of PA producing plants were unable to synthesize alkaloids.[93] Recent experiments localizing homospermidine synthase as the first pathway specific enzyme in the roots of *Senecio vernalis* revealed a highly specific expression in groups of specialized cells of the endodermis and the neighboring cortex parenchyma directly opposite the phloem,[94] the vascular tissue in which the PA-backbone structure (*i.e.*, senecionine *N*-oxide in the case of many *Senecio* species) is transported to the shoot.[62,95] In the shoot, senecionine *N*-oxide is efficiently modified by simple one- or two-step reactions, such as hydroxylations, epoxidations, dehydrogenations, and O-acetylations yielding the species specific alkaloid pattern.[96] These steps are genetically-controlled and catalyzed by specific enzymes.[97] Within *Senecio*, PAs do not show any turnover, but are steadily redistributed within the plant,[96, 98] and stored in high amounts in the inflorescences and in peripheric tissues of the stems.[99] The synthesis of the PA backbone structure is highly conserved among species producing senecionine-like PAs, and it is well protected from herbivore attack in the roots. In contrast, the diversification of this backbone structure in the shoot is highly plastic. Any genetic variation can modify the PA pattern in the plant but not the overall quantity. Indeed, it was shown that the intraspecific PA-pattern among populations is extremely variable,[100, 101] enabling the plant to cope with the demands of a continuously changing environment.[102]

The occurrence of PAs is sporadically restricted to the angiosperms in unrelated taxa,[91] as is also the case of nicotine, purine alkaloids, and tropane alkaloids.[103-105] Major occurrences of PAs are in the tribes Senecioneae and Eupatorieae within the Asteraceae, many species within the Boraginaceae, the genus *Crotalaria* within the Fabaceae, and some taxa of the Apocynaceae and Orchidaceae. In other plant families, only isolated occurrences in a few species are known (Celastraceae, Convolvulaceae, Ranunculaceae, Rhizophoraceae, Santalaceae, and Sapotaceae).[91] Such a scattered occurrence raises the question of the biosynthetic pathway origin. Is the ability to produce PAs an old evolutionary heritage that arose only once and was successively lost several times in different lineages, or did it arise several times independently and develop by convergent evolution? Answers to such questions may be possible with a more detailed understanding of the enzymes involved in PA biosynthesis.

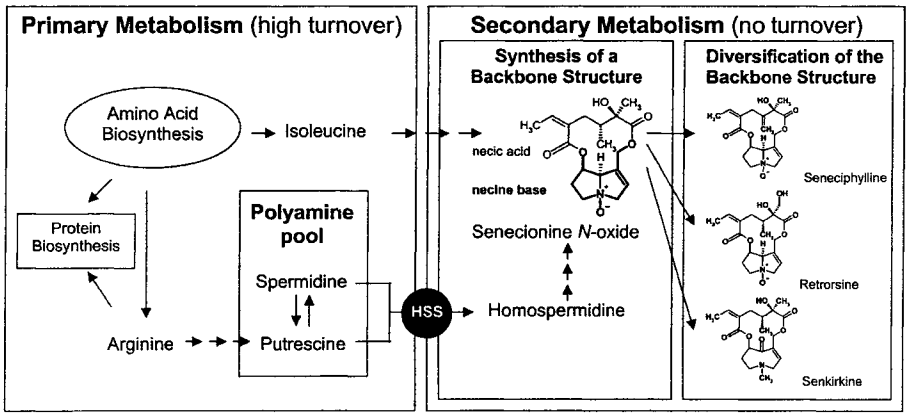

Figure 9.2. Metabolism of pyrrolizidine alkaloids (PAs) in *Senecio vernalis*. The substrates for alkaloid biosynthesis, putrescine and spermidine, are derived from primary metabolism. Homospermidine, synthesized by homospermidine synthase (HSS), is the first pathway specific intermediate. It is exclusively incorporated into the necine base moiety of senecionine *N*-oxide, the backbone structure of all PAs found in this *Senecio* species. During allocation from the roots as site of synthesis to the shoots, it is chemically modified to provide the species specific PA-pattern.

The biosynthetic pathway leading to the necine base moiety was studied intensively in the past. Early tracer experiments by Robins and coworkers revealed that the biogenetic precursors are putrescine, spermidine, arginine, and ornithine.[106, 107] The latter was shown to be incorporated only via arginine.[108] By using the diamine oxidase inhibitor β-hydroxyethylhydrazine, homospermidine was identified as the first pathway-specific intermediate.[29] The formation of homospermidine is catalyzed by homospermidine synthase (HSS), an enzyme that transfers the aminobutyl moiety of spermidine to a putrescine molecule. HSS was identified and characterized from a PA-producing plant.[109] The mechanism as well as the major molecular and kinetic properties of the plant enzyme are similar to the bacterial homospermidine synthase that has been purified and cloned from *Rhodopseudomonas viridis*.[110, 111] Both enzymes aminobutylate putrescine to form homospermidine, and use NAD^+ catalytically by transferring reduction equivalents transiently to the NAD^+.[109] The only obvious difference is the peculiarity of the bacterial enzyme that accepts as an amino butyl donor not only spermidine but also

putrescine. Molecular cloning of homospermidine synthase from the PA producing plant *Senecio vernalis* revealed no sequence similarity to the bacterial enzyme but high amino acid identity to an enzyme of primary metabolism, deoxyhypusine synthase (DHS).[112] DHS is conserved within eukaryotes and archaebacteria and is involved in the post-translational activation of the translation initiation factor 5A (eIF5A).[113,114] Although the mode of action of this regulatory protein is not well understood, it has been shown that it is essential for cell viability and cell proliferation.[115-117]

Detailed comparison of the properties of HSS of *Senecio vernalis* with DHS of *S. vernalis* and tobacco has revealed that both enzymes share the same catalytic mechanism. The aminobutyl moiety of spermidine is transferred by HSS to a putrescine molecule to form homospermidine and to a specific protein bound lysine residue by DHS to form the rare amino acid deoxyhypusine.[112, 118] Furthermore, DHS is not only able to catalyze the post-translational modification of the 18 kDa eIF5A protein as its inherent property but also catalyzes the formation of homospermidine.[112,118] The HSS, in contrast, is unable to transfer the aminobutyl moiety to the protein-bound lysine residue to modify the eIF5A precursor protein.[112] Thus, plants producing PAs must have recruited during evolution a gene copy of *dhs* for the first specific step in PA biosynthesis.[38] Mechanistically this recruitment did not require substantial modifications, because recent kinetic analyses have shown that DHS only lost its ability to bind the eIF5A on its surface. An optimization of the HSS-activity was not necessary (Ober, Harms Witte, Hartmann, unpublished results). From the evolutionary point of view, this gene recruitment is remarkable. The high degree of conservation that DHS shows within eukaryotes and archaebacteria reflects the high stringency of selective control that acts upon this essential enzyme, which has been found to date, in all completed genome projects, to be a single copy gene. A new selection pressure on the plant arising from herbivory probably had to "pull out" a gene copy of *dhs*, away from its original function in primary metabolism. Instead of activation of an essential regulatory protein, this gene copy became involved in the synthesis of low molecular weight defense compounds. We are far from understanding how such a recruitment is managed with respect to its integration into a completely new regulative network.

Concerning the scattered occurrence of PAs within angiosperms, the sequence information for HSS and its ancestor DHS may allow to answer the question of whether PA biosynthesis is of monophyletic or polyphyletic origin. Preliminary data suggest that HSS was recruited several times independently from DHS in angiosperm evolution (Ober, *et al.* unpublished results). Thus, the integration of the *dhs* gene copy into the new metabolic environment of alkaloid biosynthesis appears to be a convergent process. The origin of the enzymatic activity of HSS is on the other hand monophyletic, since this activity was already a silent activity of DHS before gene recruitment.[118] Recent, in-depth analysis of a broad selection of plants has revealed small but substantial amounts of homospermidine in all of them.[119] In

the past, homospermidine was thought to be an uncommon polyamine with limited distribution in all organistic kingdoms.[120-127] Probably, homospermidine can be regarded as by-product of universal DHS.

INSECT STRATEGY FOR ADAPTATION

Insects that recruit plant defense for their own protection are promising candidates for understanding the specific mechanisms that insects have evolved to cope with alkaloid-mediated plant defense. Several fascinating examples of alkaloid-recruitment have been described for the pyrrolizidine alkaloid system, some of which are given below. Most likely this is due to the unique feature of these alkaloids to exist in two interchangeable forms, as *N*-oxide or as free base (tertiary alkaloid form).

Two Faces of Pyrrolizidine Alkaloids

Many classes of alkaloids are able to form *N*-oxides,[128] but to occur predominantly as *N*-oxides seems to be a unique feature of PAs (Fig. 9.3).[91] With a few exceptions,[129] plants synthesize PAs exclusively as *N*-oxides.[91] The *N*-oxide is, due to its salt-like property, water soluble so it is well suited for long distance translocation and accumulation in the cell vacuole. Specific carriers, as demonstrated with cell suspension cultures of *Senecio*, mediate the specific membrane transport into the vacuole.[130] PA *N*-oxides are easily reduced to the respective free bases during extraction procedures and also in the gut of not adapted herbivores feeding on PA *N*-oxide containing plants.[48] The tertiary form of a PA in its unprotonated state can passively permeate membranes.[48] Resorbed by vertebrate and insect herbivores, this tertiary PA will be bioactivated by cytochrome P450 oxidases of the xenobiotic-metabolism to highly reactive pyrroles that are responsible for the hepatotoxic, pneumotoxic, and genotoxic effects of PAs.[131-134] However, in order to be bioactivated after reduction to their tertiary form, PAs have to fit certain structural requirements, which are met by most natural occurring PAs: (a) a necine base with a 1,2-double bond, (b) esterification of the allylic OH group at C-9, and (c) a free or esterified second OH group. Thus, PAs are *per se* not toxic. If the PA *N*-oxide, which is not a substrate for bioactivating P450 monooxygenases, is reduced to its tertiary form, it becomes a pre-toxin, because now it may be converted into toxic specimens by "failure" of the microsomal biotransformation system.

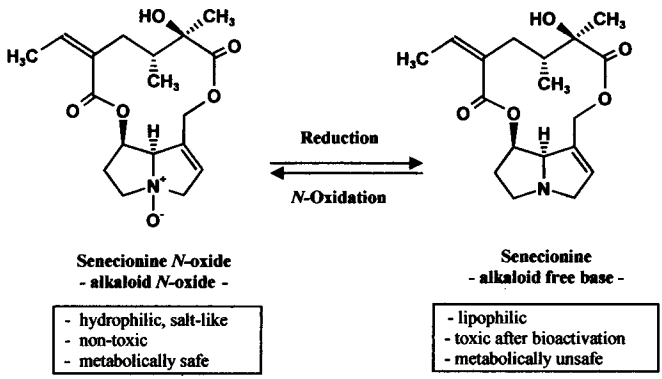

Figure 9.3. The two faces of pyrrolizidine alkaloids (PAs). The non-toxic PA *N*-oxide is easily converted into the pro-toxic free base in the gut of herbivores or predators.

Mechanisms of PA Sequestration for Insect Defense

PAs are known to be strong insect antifeedants. Several examples are found in the literature that describe deterrent activity of PAs against insects.[42, 43, 63, 135] A convincing example for feeding deterrency of PAs is given by the behavior of the giant orb spider (*Nephila clavipes*) that cuts out any PA-storing butterfly unharmed from its web, whereas freshly emerged Ithomiinae butterflies that are still devoid of PAs are readily eaten.[136-138] Due to the "powerful quality" of PAs as defense components, there are several insects which have developed mechanisms to acquire them from plants and safely store them in their own body. These insects are generally brightly colored to signal their unpalatabilty to potential predators. To date, three completely different mechanisms have been identified for safe handling and storage of plant derived PAs. Two of these are realized within the leaf beetles (Chrysomelidae, Coleoptera), and one is found mainly within the tiger moths (Arctiidae, Lepidoptera).

Oreina leaf beetles (Chrysomelidae, Coleoptera) synthesize cardenolides as part of their defensive secretions that are released from specialized exocrine glands.[139,140] Some *Oreina* beetles sequester and secrete PAs, which are taken directly as *N*-oxides from their Asteraceae food plants.[59] It is assumed that PA acquisition evolved in species that already possessed the ability to synthesize and store cardenolides for efficient defense.[141] *O. cacaliae* is the only species in this family that lost the ability to synthesize cardenolides autogenously. Instead the plant-derived PA *N*-oxides are stored in the body (primarily in the hemolymph) and

in exocrine defense glands that secrete the defense secretion in case of predation. The mechanism of PA *N*-oxide uptake into the defense glands without intermediate reduction of the alkaloid in the gut is highly specific. Only PA *N*-oxides present in the host plant are transmitted to the defense glands and are accumulated in concentrations that are 50 to 200 fold higher (100-300 mM) than in the hemolymph.[60, 142] The hemolymph acts as a kind of reservoir for refilling the defense glands after defense secretions are released.[61] Free bases of host-plant PAs that are absorbed into the hemolymph are detoxified by glucosylation.[59] *Chrysolina coerulans*, a close relative of *O. cacaliae* that feeds on PA-free mint plants, is unable to sequester ingested PA *N*-oxides, but reduces them in the gut as do other non-adapted herbivores, supporting the specificity of mechanism for PA *N*-oxide sequestration in *O. cacaliae*.[59]

Platyphora leaf beetles belong to the same subfamily (Chrysomelinae) as *Oreina* leaf beetles, but release, instead of cardenolides, pentacyclic triterpene saponins from their defense glands that are probably synthesized autogenously from plant-derived precursors.[143-145] Like *Oreina*, within the genus *Platyphora* several species have evolved the ability to sequester and to release PAs derived from their food plants.[141,146] In contrast to *Oreina*, however, PAs are not stored as their *N*-oxides but rather as tertiary alkaloids. Thus, ingested PA *N*-oxides that are reduced in the gut of this herbivore are resorbed and directly transferred to the defense glands in their tertiary form.[147] This mechanism has to be very efficient, since in the defense glands, PAs are accumulated in concentrations up to 33-38 mM, while the hemolymph remains almost free of them.[147, 148] Larvae of *Platyphora* that are devoid of defense glands are also able to accumulate tertiary PAs in their body.[149] Thus, some aspects of PA sequestration by *Platyphora* remain puzzling. How do the larvae prevent bioactivation of tertiary PAs in their body? How are the lipophilic PAs that easily permeate membranes stored so efficiently in the defense glands? In any case, it seems obvious that due to the different biochemical mechanisms of PA uptake and maintenance in *Oreina* and *Platyphora*, the phenomenon of PA-sequestration must be of independent evolutionary origins.[147]

A third strategy for coping with accumulated PAs is realized by larvae of Arctiidae moths. As in unadapted herbivores, PA *N*-oxides present in the larval diet are efficiently reduced in the gut. Due to their lipophilic properties, these alkaloids are easily resorbed into the hemolymph. To detoxify and to trap them in a non-toxic, hydrophilic form they are efficiently *N*-oxidized in the larval hemolymph and subsequently distributed to all tissues. Based on feeding experiments with senecionine [^{18}O]*N*-oxide, an *N*-oxide-specific carrier for the direct uptake of plant derived *N*-oxides into the hemolymph was excluded.[48] All labeled senecionine was recovered in the hemolymph as [^{16}O]*N*-oxide. The same phenomenon was demonstrated for the grasshopper *Zonocerus variegatus* (Orthoptera).[48]

Larvae of the Arctiidae are able to locate PA-containing plants. *Creatonotos transiens* larvae, for example, are strongly attracted by PAs that act as strong

feeding-stimulants. Even glass-fiber discs impregnated with PAs are readily consumed by the larvae.[63] Probably, the original signal mediating feeding-deterrence was converted to a signal that stimulates feeding during adaptation to PA-containing plants. All PAs taken up by the larvae are retained during metamorphosis to the adult stage.[97] While the female adult transfers most of its PA load directly to the ovaries and subsequently to the eggs, the males of *Creatonotos transiens* or *Utetheisa ornatrix*, for example, use part of the PAs to synthesize their specific PA-derived pheromones to attract females for mating.[150, 151] The males use these pheromones to signal the female the amount of PAs they are able to transfer by the spermatophore as a nuptial gift, thus, they also participate in the protection of the eggs.[57, 152, 153] It has been shown that the PA content of the eggs provides efficient protection against ants or coccinellid beetles.[153,154] Some arctiids are even able to synthesize insect specific PAs by using the necine base moiety of the hydrolyzed plant-derived PAs.[150,155,156] Insect PAs can account for a considerable proportion of the alkaloid load of an individual.[97]

The enzyme responsible for the *N*-oxidation of PAs in the hemolymph of arctiids has been studied in detail with *Tyria jacobaeae*.[48] An enzyme called senecionine *N*-oxygenase (SNO) was purified from collected hemolymph and was shown to be a soluble flavin dependent monooxygenase that is strictly NADPH-dependent. Analysis of its substrate specificity revealed that only PAs with exactly those features that mediate the toxicity of PAs (see above) are *N*-oxidized.[48] Other xenobiotics or related alkaloids were not accepted as substrates. Thus, only toxic PAs are trapped in their non-toxic *N*-oxide form to guarantee optimal defense of the insect. Flavin dependent monooxygenases (FMOs) are well characterized from xenobiotic metabolism of vertebrates. Such a FMO has already been mentioned as accounting for the resistance of guinea pigs to PAs. In contrast to the SNO of *Tyria*, the microsomal enzymes are characterized by a broad substrate specificity to allow these phase I detoxification enzymes to *N*-oxidize a wide array of potential xenobiotics.[157,158]. In insects, cytochrome P450 dependent monooxygenases are the most important enzyme system dealing with plant derived toxins[159] and also with many man made insecticides.[160,161] Postulated FMOs have never been found in insects.[162,163]

Recent molecular cloning of *Tyria* SNO resulted in a cDNA containing the putative binding sites for FAD and NADPH[164] that are common within FMOs.[157] Contrary to mammalian FMO, the SNO lacks hydrophobic transmembrane anchor motifs and contains an N-terminal signal peptide characteristic for extracellular proteins.[164] Comparative sequence analysis of FMOs present in the database revealed phylogenetic relationships among FMO-like sequences from mammals, nematodes, fungi, and plants (Fig. 9.4). Except for the mammalian FMOs, most of these sequences are the result of genome projects and await functional characterization. For the FMO-like gene of yeast, it has been shown that it might be involved in maintaining the optimal redox environment in the cell for proper folding of disulfide

containing proteins.[165] A similar role was postulated as the original function of mammalian FMOs.[166] Whether redox regulation is also the physiological role of two functionally uncharacterized drosophilid genes that resulted from the *Drosophila* genome project remains open. Probably one ortholog of these *Drosophila* genes is or was present in earlier times in the genome of *Tyria jacobaeae* sharing a common ancestor with SNO.[164] The functional characterization of FMO-like sequences will be a major challenge for the future. FMO-like sequences reflect a mainly uncharacterized gene family with 29 homologues in the genome of *Arabidopsis thaliana* alone. For only one of them, the involvement in auxin biosynthesis has been postulated,[167] as it was also for an homologue of *Petunia*.[168]

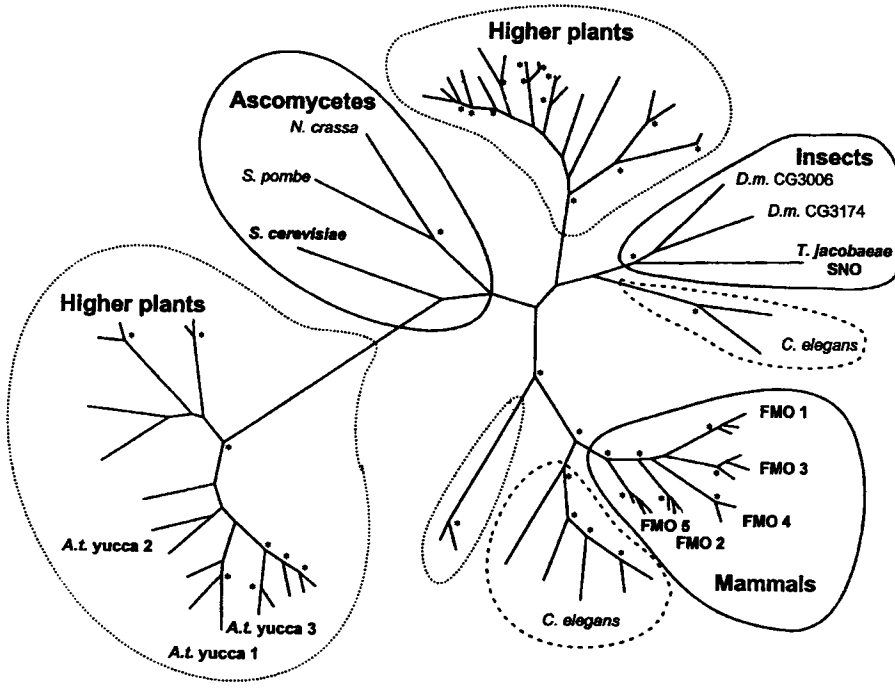

Figure 9.4. Phylogenetic relationships among proteins supposed to be related to senecionine *N*-oxygenase of *Tyria jacobaeae*. Sequences were found in different databases and resulted mainly from different genome projects. Branches with bootstrap support of ≥ 0.9 are indicated by an asterisk.[164]

SUMMARY

Research in life science has entered the "omics" era. Terms like genomics, proteomics, metabolomics, or transcriptomics are used almost everywhere to explain the problems evolving from the genome projects: we know a huge number of genes from microorgansims, plants, and animals, including man, without having an idea about the physiological importance of most of them.[169] This applies even more for plant secondary metabolism. Without knowledge of the main functional aspects, these approaches will be only of limited help in understanding the molecular and genomic basis of plant secondary systems such as alkaloid metabolism. If the gene coding for homospermidine synthase as the first specific enzyme in pyrrolizidine alkaloid biosynthesis had emerged from a genome project, most probably it would have been classified, due to sequence homologies, as a functionless deoxyhypusine synthase. Who could have characterized the specific senecionine *N*-oxidase of *Tyria jacobaeae* without knowing the functional aspects of pyrrolizidine alkaloid *N*-oxidation in this adapted insect? The field of chemical ecology offers many fascinating examples of interactions of a plant with its environment. These functionally well characterized systems are promising starting points for elucidating the molecular and genetic mechanisms that have evolved in plants under the selection pressures of a hostile environment, or, for example, in herbivorous insects as a consequence of adaptation. We now have the tools to trace the origin of genes involved in such highly adaptive systems.

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Chapter Ten

THE CHEMICAL WIZARDRY OF ISOPRENOID METABOLISM IN PLANTS

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INTRODUCTION

Mention terpenes, or more properly isoprenoids, to a biologist and they are likely to conjure up mental images of chemical structures with a striking similarity to hexa-methyl chicken wire. Mention the same to a chemist and they are likely to imagine swirling electrons and carbocations within the complexities of the chicken wire. Yet, everyone is familiar with terpenes. These are the compounds that give many of our foods wonderful aromas and tastes, and the fresh scents to many of our household cleaning products. They include pinenes (pine scent) and nootkatone (distinctive grapefruit taste) (Fig. 10.1). These are some of the obvious ways we have taken advantage of these compounds. Biologically, they are immensely important compounds in plants, fungi, bacteria, and insects, which provide unique means for these organisms to sense and interact with their environment, as well as to serve as internal signals coordinating developmental programs. Two recent examples from the literature help to illustrate this point.

ISOPRENOIDS ARE IMPORTANT FOR PLANTS AND MAN

Wagner and co-workers have had a long standing interest in leaf surface chemistry and in particular the diterpene constituents that are synthesized by tobacco leaf hairs or trichomes.[1] Earlier studies hinted at the possibility that several leaf surface compounds and especially the cembratriene alcohols (diterpenes) (Fig. 10.2) might serve as deterrents to insect pests such as aphids.[2] Using a P450 gene that was isolated on the basis of its exclusive expression in trichomes and a suspicion that it encoded a terpene hydroxylase, Wang *et al.* engineered both sense and anti-sense constructs of this gene under the direction of a strong constitutive promoter into transgenic plants.[3] The investigators observed that at least one of the anti-sense transgenic lines and two of the sense (co-suppressed) transgenic lines had reduced levels of the P450 mRNA level, which corresponded to an approximate 40% decrease in the cembratriene-diol levels. These lines also had greatly enhanced levels of the cembratriene mono-alcohols, the direct precursors to the cembratriene-diols. The suppression of diol levels in the transgenic lines did not effect any morphological traits, except that the transgenic lines were not colonized by aphids to the same extent as control plants. The reduced colonization was not because the aphids were less attracted to the transgenic lines, but because of an enhanced toxicity of the transgenic lines to colonizing aphids, due to the increased levels of the mono-hydroxylated cembratrienes.

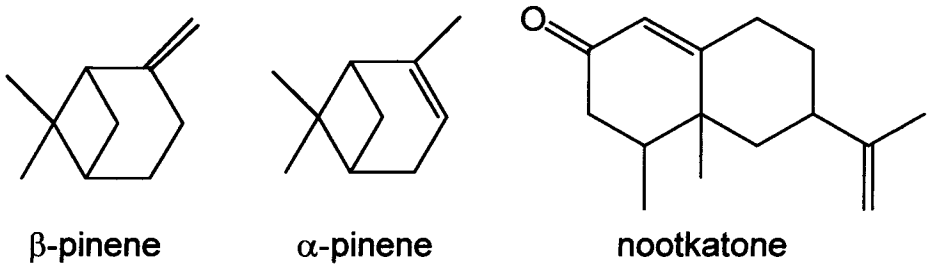

Figure 10.1: Structures of terpenes having strong fragrances and flavors.

Assessing the biological significance of any natural product to a plant's fitness has been a particular important challenge that often has focused on so-called inducible defense compounds. Because one of the best characterized responses of plants to microbial challenge is the production of phytoalexins, tremendous experimental work has been directed at evaluating these responses, and calculating the contribution of a particular inducible defense compound to the plant's defense capabilities.[4,5] Plants, however, are also known to accumulate a variety of secondary metabolites possessing antimicrobial activities during the normal course of growth and development. Although there has been some progress in assessing the contribution of "phytoanticipins" to plant traits, the contribution of these pre-formed defense compounds to a plant's susceptibility or resistance to microbial pathogens has been understudied.[6] This deficiency has recently been addressed in a series of decisive reports .

Osbourn and her colleagues previously determined that a fungal pathogen of *Avena* species was considerably more pathogenic if it expressed an enzyme capable of detoxifying avenacins, the triterpene saponins associated with oat roots.[7] A more direct assay for how important the oat-derived avenacins were for resistance, however, was lacking. Using the diploid species *Avena strigosa*, this research group developed a clever fluorescence screen to isolate chemically induced mutants that did not accumulate avenicins (*sad* mutants for saponin-deficient) (Fig. 10.3), and these mutants were then examined for disease severity after challenge by a fungal pathogen for a specified length of time. In general, Papadopoulou *et al.* observed that *sad* mutants with reduced avenacins were more susceptible, and that the reduced avenacin content and disease susceptibility traits co-segregated in F2 progeny arising from crossing wildtype and mutant plants.[8] Several of the *sad* mutants have subsequently been mapped to mutations within a novel triterpene cyclase correlated with avenacin biosynthesis.[9]

Given the biological activities of terpenes, it is not surprising that many have significant practical applications. The diterpene Taxol[tm] is now considered one of the most efficacious agents for a range of cancer types,[10] and artemisinin, a sesquiterpene, is a promising lead agent for the treatment of malaria.[11] Agricultural uses for terpenes include insect and microbe control agents[12] and additives for many food applications.[13] Terpenes are also used in industrial lubricants and as paint additives.[14,15]

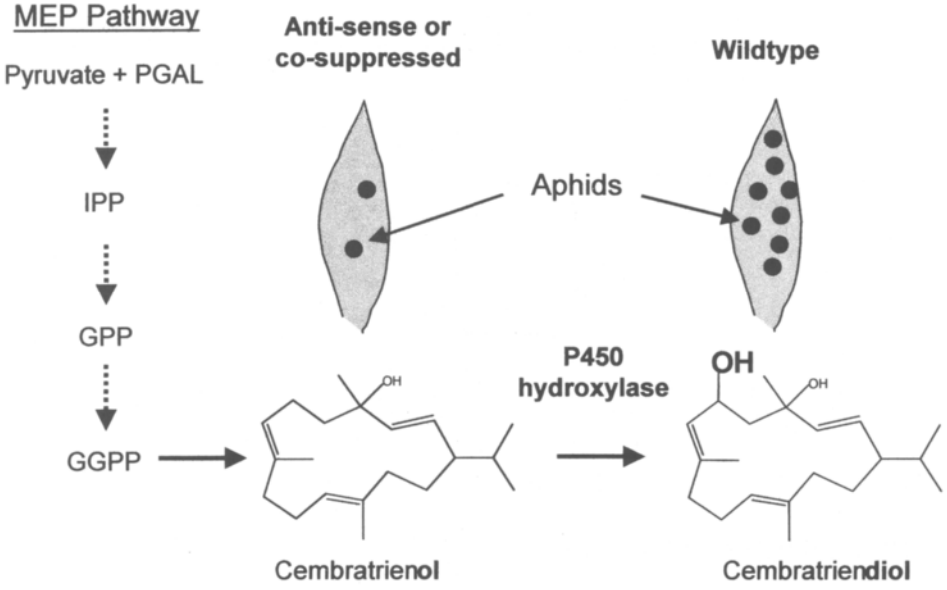

Figure 10.2: Anti-sense or co-suppression of a putative P450 enzyme involved in diterpene metabolism in tobacco results in reduced levels of cembratriene-diols, increased levels of cembratrien-ol, and decreased colonization by aphids.[1]

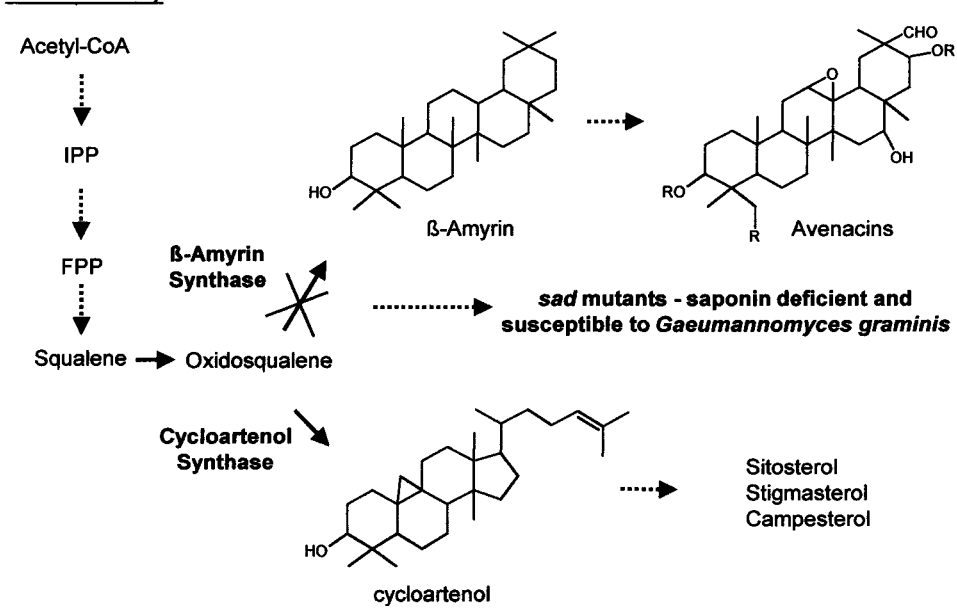

Figure 10.3: *Sad* mutants of oats, *Avena* spp., are deficient in their ability to synthesize anti-microbial saponins and are susceptible to normally avirulent fungi.[8,9]

THE ISOPRENOID PATHWAYS

One of the more exciting and recent advances in the field of plant biochemistry has been the discovery of the mevalonate-independent pathway for the biosynthesis of isoprenoids (Fig.10.4). This new pathway, referred to a the methyl-erythritol-phosphate or MEP pathway for the first intermediate committed solely to the biosynthesis of isoprenoids, was first discovered in prokaryotes capable of accumulating hopenes, the equivalent of eukaryotic sterols.[16,17] The MEP pathway has since been confirmed in plants and, not surprisingly, has been localized to chloroplasts.[18] Operation of the MEP pathway is intimately related to the reactions of CO_2 fixation and photosynthesis, as evidenced by the two immediate precursors pyruvate and phosphoglyceraldehyde for this pathway. Two important features of this pathway are that mevalonate is not an intermediate in the plastidic synthesis of isopentenyl (IPP) and dimethylallyl diphosphate, (DMAPP), and this pathway

appears to be largely responsible for the biosynthesis of monoterpenes, diterpenes, tetraterpenes (carotenoids), and polyprenols. Sesquiterpenes, sterols, and triterpenes are synthesized in the cytosol via the mevalonate pathway (for the most part[19]), which is responsible for the synthesis of the key intermediate farnesyl diphosphate (FPP). Hence, specific classes of isoprenoids are derived from the independent operation of two very different pathways (Fig. 10.4).

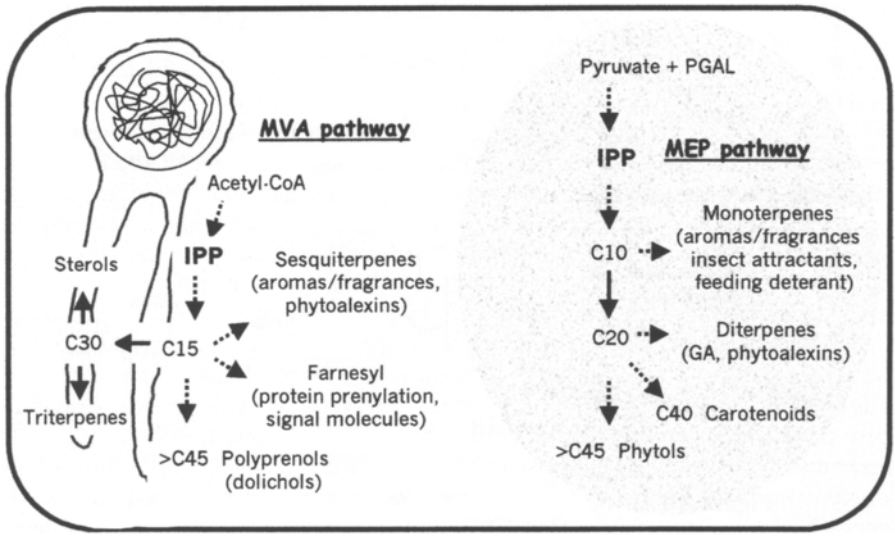

Figure 10.4: Plants have mastered two pathways for terpene/isoprenoid biosynthesis. The mevalonate pathway is specialized for the biosynthesis of 15 and 30 carbon compounds in the cytoplasm, while the methyl-erythritol phosphate pathway is specialized for 10 and 20 carbon compounds produced in the chloroplasts.

SYSTEMS FOR STUDYING ISOPRENOID METABOLISM

Investigations of isoprenoid metabolism and biochemistry in plants have been hampered for several reasons. Some isoprenoids accumulate over long developmental time courses, which suggests that the enzymes responsible for their biosynthesis are either present in low abundance or have low activity levels. Sterols

are an excellent example of this point. Although sterols might accumulate to 0.1-0.4% of a leaf's dry weight, this accumulation occurs over the lifetime of a leaf.[20] Other classes of isoprenoids are also synthesized in low amounts in specific cell types or tissues. Such low abundance isoprenoids include monoterpenes, sesquiterpenes, and diterpenes synthesized in glands, ducts and trichomes.[21] To make matters more complex, some isoprenoids only accumulate in response to specific environmental cues, such as light conditions or in response to pest/microbial challenge.[22]

Plant cell cultures have proven useful in overcoming several of these limitations. For example, when fungal elicitors are added to rapidly growing tobacco-cell suspension cultures, the cultures cease sterol production and instead synthesize and secrete antimicrobial sesquiterpenes (Fig. 10.5).[23] The decline in sterol biosynthesis has also been correlated with a suppression of squalene synthase enzyme activity, and the induction of sesquiterpene accumulation correlated with the induction of a sesquiterpene cyclase enzyme activity.[23] Because these two enzymes are positioned at a putative branch point in the isoprenoid pathway, the induction of one enzyme and the suppression of the other have been interpreted as important mechanisms controlling carbon flow and hence, end-product formation (Fig. 10.6). This rapid changeover in metabolism, within 12 hours of elicitor treatment, and the relative ease of propagating large volumes of elicitor-treated cell cultures facilitated efforts to purify several of the key enzymes,[24] to isolate and characterize cDNAs for these particular enzymes,[25] and provided opportunities to investigate the regulatory mechanisms controlling this biosynthetic machinery.[26] In short, induction of the sesquiterpene branch pathway appears to be regulated predominately by transcriptional control mechanisms,[25,26] while the suppression of sterol biosynthesis is subject to transcriptional and post-translation control mechanisms.[27,28]

BIOCHEMICAL COMPLEXITIES AND CHEMICAL WIZARDS

What makes terpenes so unique is the chemical wizardry nature has evolved in order to produce them (Fig. 10.7). Of the thousands of natural products identified to date, approximately one quarter to one half are estimated to be terpenes, and various calculations suggest that this is only 1-10% of the diversity likely to be found in nature. The rationale for such calculations is based in large part on the nature of their biosynthesis. The more than 30,000 different compounds identified are derived largely from three biosynthetically related, yet simple looking precursors.[29] These precursors, geranyl diphosphate (GPP), farnesyl diphosphate (FPP), and geranylgeranyl diphosphate (GGPP), are essentially polymers of 2, 3, or 4 isoprene groups (5-carbon alkene units) covalently tethered together and punctuated with a diphosphate. The magic begins when one starts to gaze at the amazing number

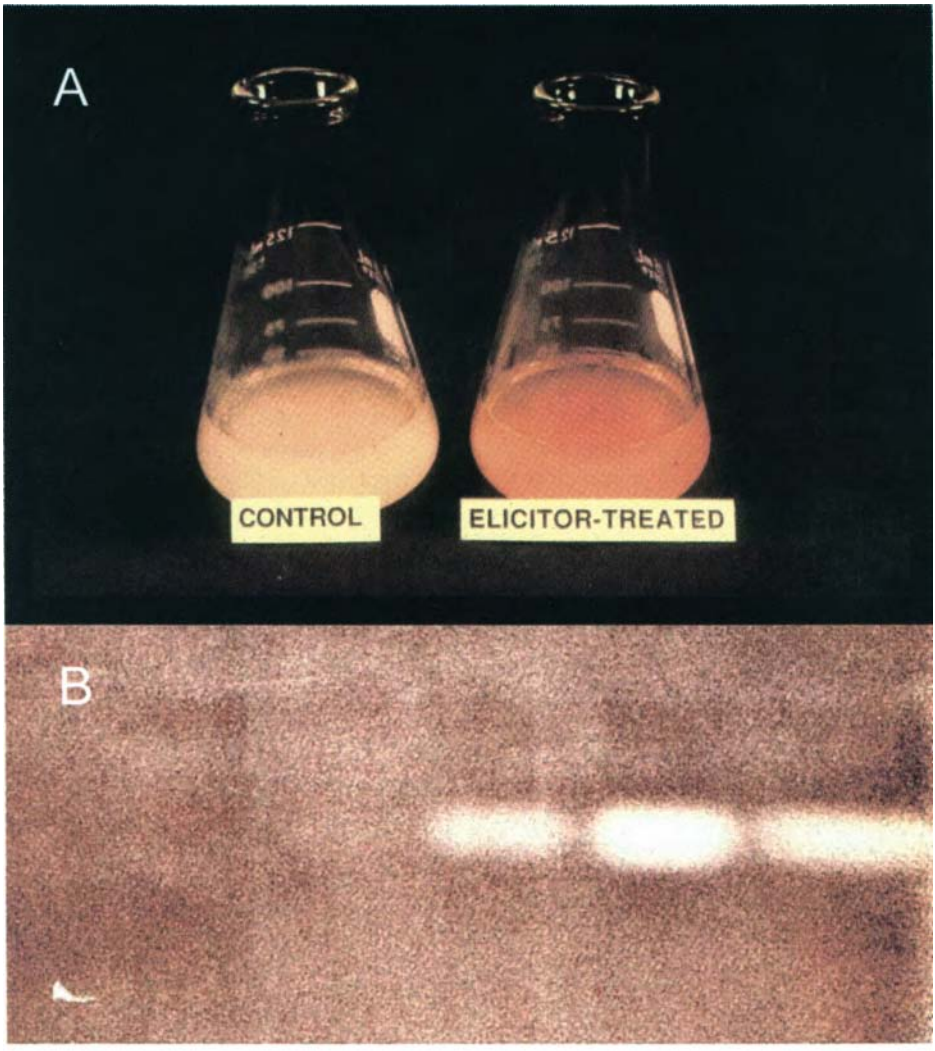
A
CONTROL
ELICITOR-TREATED
B
0
3
6
12
18
24
Time after elicitation (hr)

Figure 10.5: Plant cell cultures have proven to be very useful for studying plant-pathogen interactions and isoprenoid metabolism. Tobacco cell cultures respond rapidly to the addition of fungal elicitors (0.5 μg cellulase/ml of culture) by browning (A) (analogous to a hypersensitive response) and the production of phytoalexins (B). Media was collected from elicited cell cultures at the indicated times, partitioned against an organic solvent, and concentrated aliquots run on a silica TLC plate. The plates were then sprayed with a suspension of *Cladosporium cucumerinum* spores and incubated in a humid environment for 5 days before viewing (B). The compound released from the elicitor-treated tobacco cells that inhibits spore germination is capsidiol, a sesquiterpene.

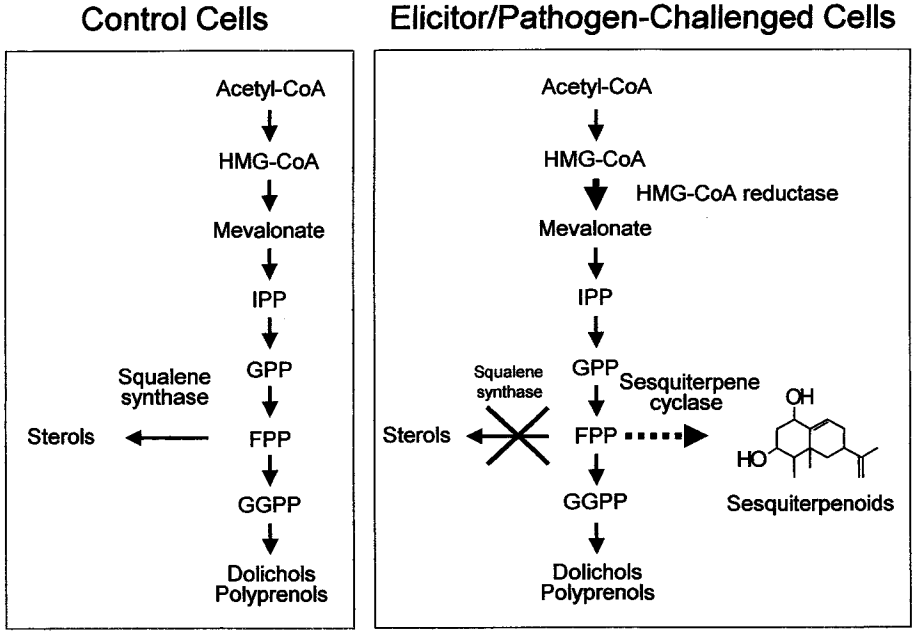

Figure 10.6: A cartoon depiction of the metabolic changes that occur in tobacco cells in response to fungal elicitor treatment.

of structural derivatives that can arise from each of these precursors (Fig. 10.8), and then considers how this diversity might arise by the action of a single biosynthetic step catalyzed by a terpene synthase (Fig. 10.9).

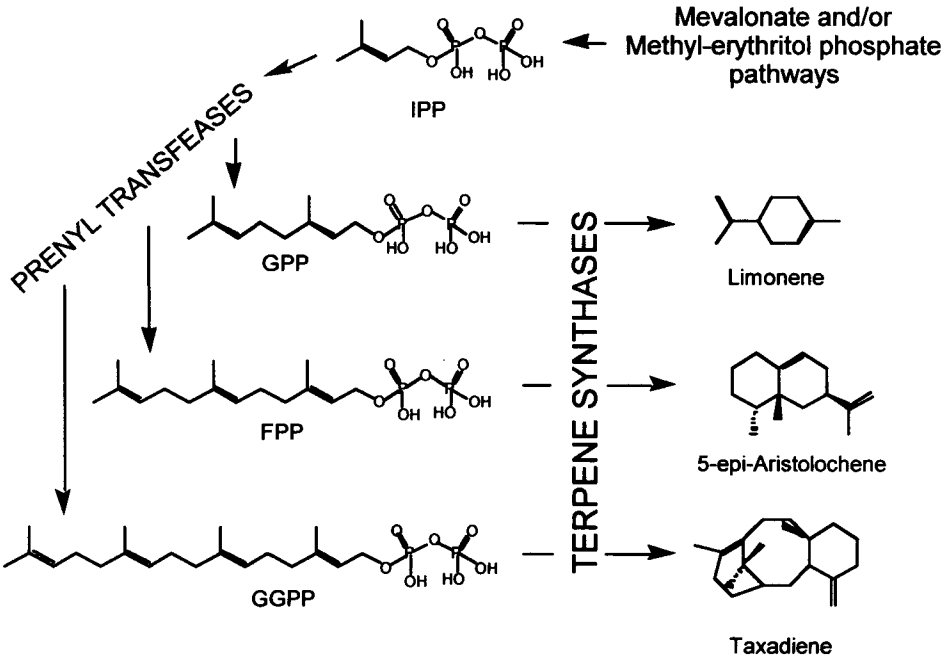

Figure 10.7: All terpenes are derived from allylic diphosphates which are polymers of repeating isopentyl units (IPP) put together by the action of prenyltransferases. In plants, IPP can be derived from the mevalonate biosynthetic pathway (a cytoplasmic pathway) or the methyl erythritol phosphate pathway (a plastidic pathway). Monoterpenes are then derived from the C10 precursor geranyl diphosphate (GPP), sesquiterpenes from the C15 precursor farnesyl diphosphate (FPP), and diterpenes from the C20 precursor geranylgeranyl diphosphate (GGPP) by the action of terpene synthases or cyclases, which divert carbon into the specific branch pathways.

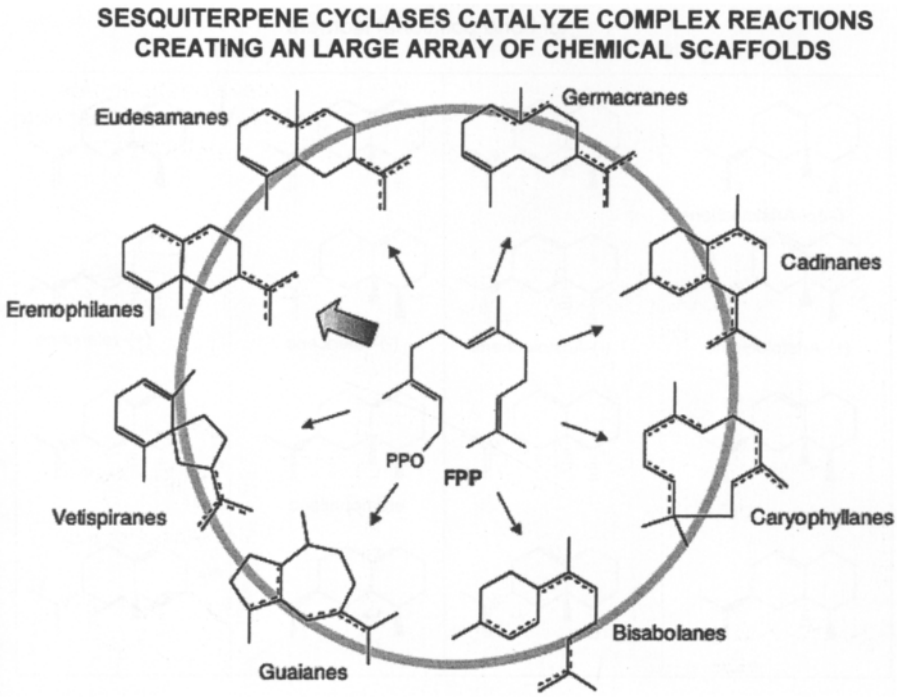

Figure 10.8: Farnesyl diphosphate (FPP) can be cyclized in many different ways by sesquiterpene synthases giving rise to different classes of sesquiterpenes. The eremophilane class of sesquiterpenes has received considerable attention because the genes coding for the corresponding synthase enzymes were the first to be isolated .[25]

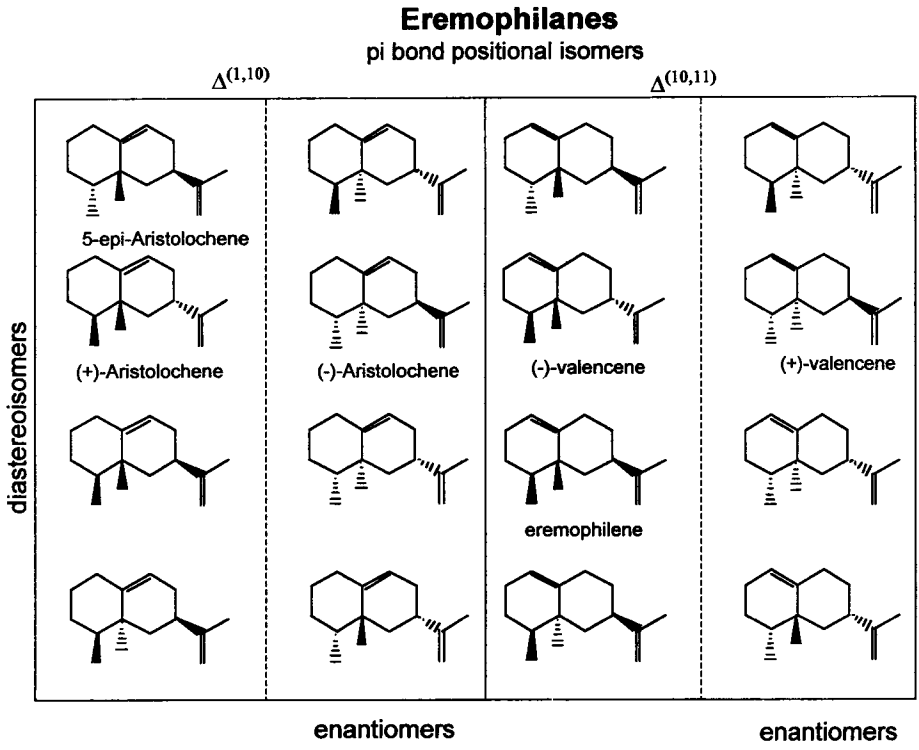

Figure 10.9: Each class of sesquiterpenes (see Fig. 10.8) consists of many structural variants that arise from stereochemical positioning of methyl groups and sites of double bond formation. Depicted are the possible variants within the eremophilane class of sesquiterpenes, which are hypothesized to arise from an equally related class of sesquiterpene synthases. Only those structural derivatives that have been found in nature or synthesized are denoted by name. The remaining structures should, therefore, be considered novel.

Although terpene synthase is the proper term for these enzymes, many investigators prefer "cyclase" because of its descriptive value. To produce the monoterpenes, sesquiterpenes, and diterpenes, the terpene cyclases bind the allylic diphosphate substrates and initiate catalysis by heterolytic cleavage of the diphosphate group to generate a reactive allylic carbocation. Next, these same enzymes must protect the reactive carbocation(s) from being prematurely quenched or lost to the bulk solvent, while simultaneously coaxing the reaction intermediates down one of an almost infinite number of reaction cascades involving 2, 3, or more partial reactions. Lastly, the terpene cyclases must release these hydrophobic reaction products into an aqueous, hydrophilic environment.

Significant progress has been made during the last decade in detailing the biochemical reactions catalyzed by the terpene synthases.[30-34] For example, hundreds of cyclase gene sequences are reported in GenBank, and a significant but much smaller number of these have been functionally expressed in heterologous hosts,[35-43] and probed for structure-function relationships with a combination of site-directed mutagenesis[32,34,44] and crystallographic studies.[45-48] Such investigations have opened the way for rational re-designing of the terpene cyclases. For example, we have used site-directed mutagenesis to alter the 5-epi-aristolochene synthase gene and have generated mutant enzymes that have catalytic constants (K_m, K_{cat}) comparable to wildtype enzyme, yet synthesize germacrene A (Fig. 10.10) (Rising *et al.*[32]; Greenhagen and Chappell, unpublished data).

ELABORATING ON A THEME

The structural variety of terpenes is further elaborated by subsequent modifications to the cyclic scaffolds or backbones by hydroxylations, methylations, demethylations, halogenations, oxidations, and possibly even glycosylations.[49] Many of these modifications are catalyzed by P450 enzymes, and several of these have been the focus of in-depth investigations lately.[50-55] For example, although the sesquiterpene cyclases responsible for the biosynthesis 5-epi-aristolochene had clearly been identified, the mechanism(s) for conversion of 5-epi-aristolochene to capsidiol in elicitor- or pathogen-challenged solanaceous plants had not until recently (Fig. 10.11). Earlier work by Whitehead *et al.* had suggested that hydroxylation at C3 occurred first by an elicitor-inducible P450-mediated enzyme, followed by hydroxylation at C1 catalyzed by a constitutive enzyme activity.[56] Hoshino *et al.* actually provided the first *in vitro* characterization of a 5-epi-aristolochene hydroxylase activity using microsomal preparations from elicitor-induced pepper.[57] The assay developed by these investigators measured the amount of 1-deoxycapsidiol generated upon incubation of pepper microsomes with 5-*epi*-aristolochene by GC analysis. Interestingly, Hoshino *et al.* reported never observing 3-deoxycapsidiol as a product, nor apparently the dihydroxylated product, capsidiol.

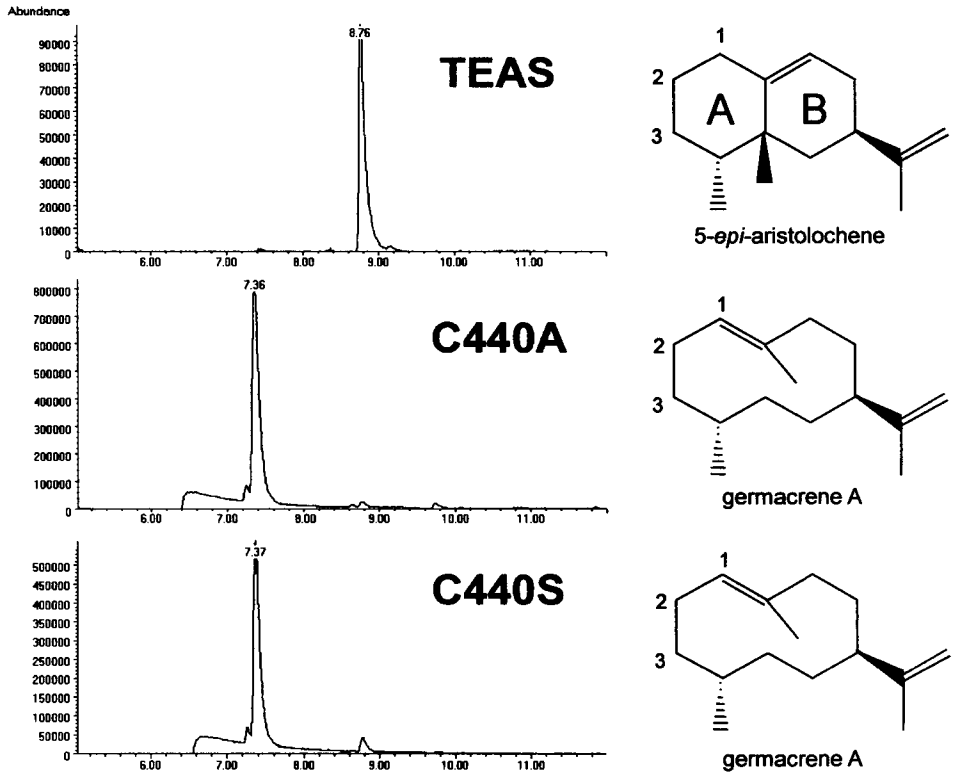

Figure 10.10: Gas chromatograms of the reaction products formed upon incubation of wildtype tobacco 5-epi-aristochene synthase (TEAS) and mutant (C440A, C440S) enzymes with FPP. The cDNA for 5-epi-arisolochene synthase was mutagenized using standard oligo nucleotide directed mutagenesis, and the respective cDNAs then expressed in E. coli. The wildtype and mutant enzymes were purified from bacterial lysates based on histidine-tags engineered at the amino terminus of the enzymes.The identity of germacrene A and 5-epi-arisolochene were verified by MS.[32,35]

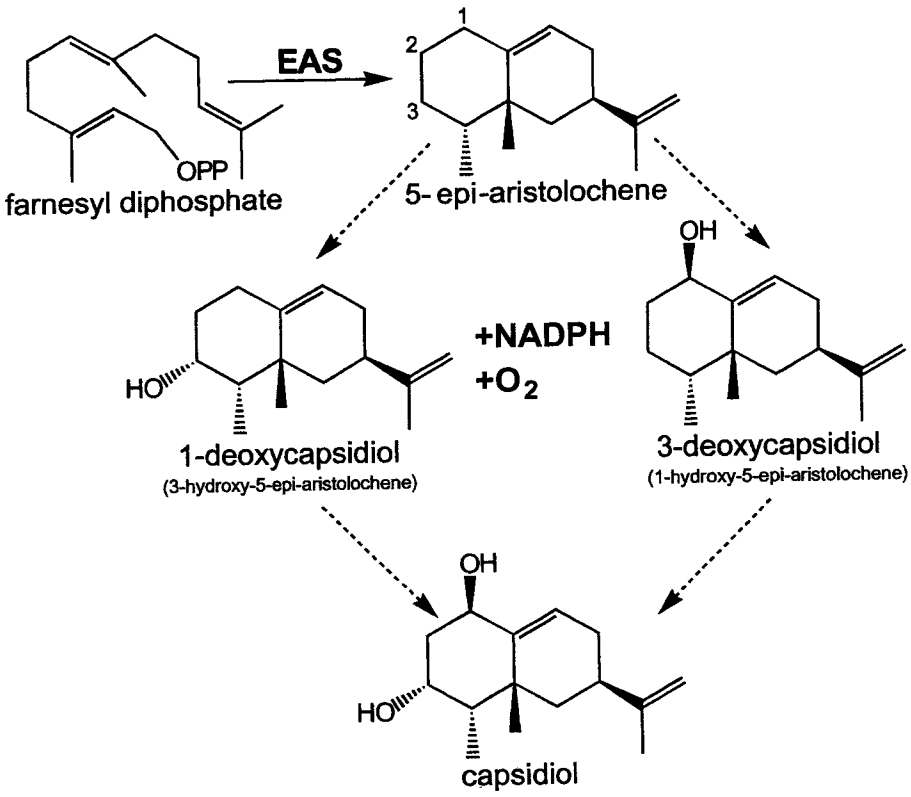

Figure 10.11: A proposed pathway for the biosynthesis of capsidiol in elicitor-treated tobacco cell cultures. Earlier work had not resolved how 5-epi-aristolochene, synthesized from FPP by the action of 5-epi-aristolochene synthase, was converted to capsidiol.

In an effort to more definitively identify the final enzymes and steps in capsidiol biosynthesis, Ralston *et al.* recently isolated a gene (CYP71D20) coding for a terpene modifying enzyme and characterized 5-epi-aristolochene dihydroxylase (EAH), a sesquiterpene dihydroxylase capable of introducing 2 hydroxyls into the 5-epi-aristolochene skeleton (Fig. 10.12).[58] While cytochrome P450 enzymes capable of catalyzing multiple reactions including sequential hydroxylations have been observed previously, the CYP71D20 hydroxylase is unique in its ability to introduce

two hydroxyl functions into a terpene ring simultaneously. The relative importance of such chemical modifications is that they often increase the water solubility characteristics of the terpenes, which in turn has also been correlated with increasing the biological activity of these compounds.[59]

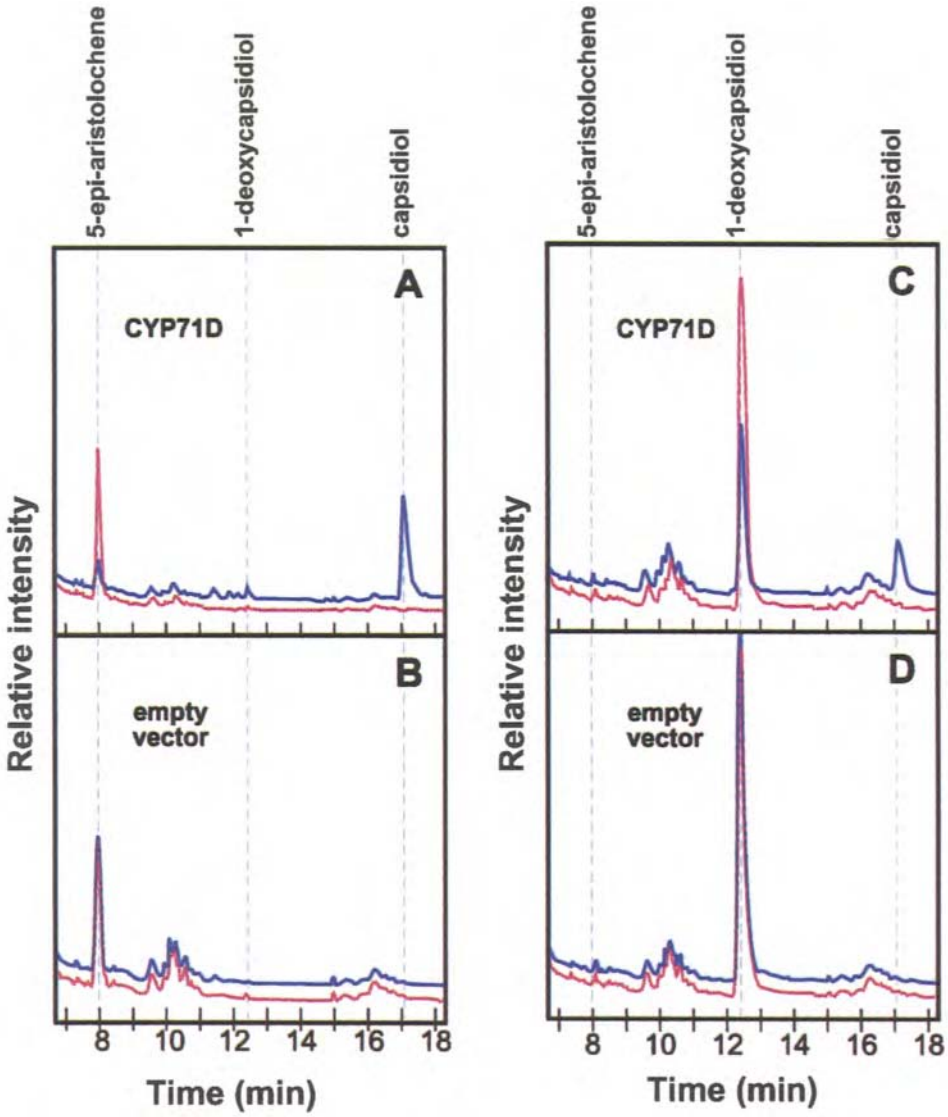

Figure 10.12: Gas chromatograms of the reaction products formed upon incubation of microsomes isolated from yeast over-expressing a tobacco terpene hydroxylase gene (panels A & C) (CYP71D20) or harboring only the expression vector DNA (control) (B & D). Microsomes were incubated with 5-epi-aristolochene (A & B) or 1-deoxycapsidiol (C & D) in the presence (blue line) or absence (red line) of NADPH. 5-epi-aristolochene, 1-deoxycapsidiol and capsidiol were all verified by MS.[58]

SUMMARY – GAZING INTO THE TERPENE CRYSTAL BALL

This chapter has attempted to give readers a general appreciation for some of the exciting research at several different levels in the field of terpene and isoprenoid metabolism in plants. Biologists are using the tools of molecular genetics to define the contribution of isoprenoids to the ability of plants to interact with their environment. Molecular biologists and geneticists are determining mechanisms regulating the expression of this biosynthetic machinery, and biochemists are elucidating structure-function relationships for key enzymes in the pathway. Bringing this array of information back together and using it for the creation of novel terpenes having unique biological roles is clearly one of the next great challenges in the field. This will include the development of designer enzymes (new catalytic functions evolved using molecular tools), the coupling of enzymes in novel ways (physically and biochemically), and expressing *de novo* isoprenoid pathways in transgenic microbes and plants.

ACKNOWLEDGEMENTS

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Chapter Eleven

THE SABATH FAMILY OF MTS IN *ARABIDOPSIS THALIANA* AND OTHER PLANT SPECIES

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INTRODUCTION

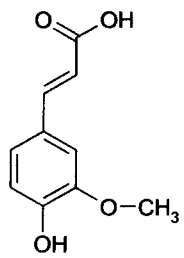
Overview of Methylation in Plant Specialized Metabolism

Methylation is one of the most common enzymatic modifications in plant specialized (secondary) metabolism. Almost all classes of plant specialized metabolites are known to be methylated, including amino acids, alkaloids, phenylpropanoids, sugars, purines, sterols, thiols, and flavonoids. The methyl transfer most commonly occurs on C, N, S, or O atoms.

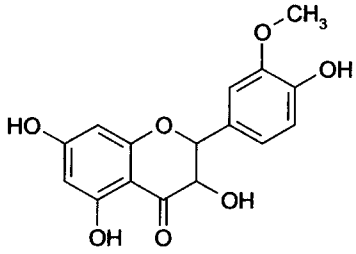
O-methyl ethers and esters make up the largest class of methylated specialized metabolites. For example, the major constituents of lignin, a phenylpropanoid polymer used for support in strengthening structural tissues and tracheary elements, consist of the alcohols derived from hydroxycinnamic acids, including the methylated compounds ferulic acid and sinapic acid (Fig. 11.1).[1,2] Some anthocyanin pigments are methylated to achieve subtle shifts in absorption spectra. Pectin derivatives are methylated polymers important for plant cell wall structure. The most common of the three classes of pectin is HGA, a homopolymer of α-1,4-linked D-galacturonic acid that can be modified by acetylation at the C-2 or C-3 hydroxyl groups and methylation at the C6 carboxyl group (Fig. 11.1).[3,4] In the walls of young plant cells, HGA has a high degree of methyl esterification. In contrast, older cells have a lower percentage of methyl esters.[5] This may be a way to give greater plasticity to the cell wall during times of growth and development. The chlorophyll pigments, essential for photosynthesis, also contain a methyl ester moiety.[6]

Methyl ethers and esters are also found as volatile constituents in a wide range of flowers and fragrant herbs. For example, methyleugenol is found in the essential oil of herbs such as sweet basil and in the floral headspace of a wide range of flowers.[7-10] Defense compounds and phytoalexins are commonly O-methylated as well. These compounds include the 3'-O-methylated form of the flavonoid quercetin as well as the 7-O-methyl ester of the flavone apigenin (Fig. 11.1).[11,12] While not within the scope of this paper, it should be noted that protein O-methylation is common.[13,14] One interesting protein modification known to occur in *Arabidopsis thaliana* is the α-carboxyl methylation of prenylated proteins.[15] This modification has been shown in mammals and yeast to be important in protein targeting and membrane association.[16,17]

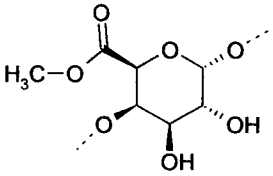
Carbon methylation occurs in the production of tocopherols, also known as vitamin E, and siroheme, a compound used as the prosthetic group for sulfite and nitrile reductases.[18,19] In addition, carbon methylation is also observed in the



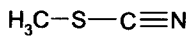
Ferulic Acid



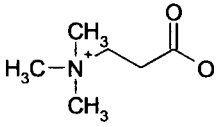
3'-Methyl Quercetin



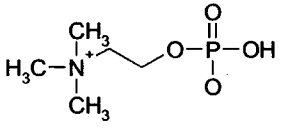
Methyl HGA subunit



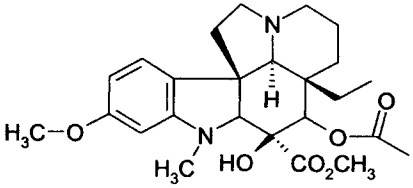
Methyl Thiocyanate



β-Alanine betaine



Phosphocholine



Vindoline

Figure 11.1: Examples of methylated plant specialized metabolites.

conversion of cycloartenol to phytosterols, compounds that regulate plant growth and proliferation.[20-22] The glucosinolates, produced mainly by species of the Brassicaceae family, provide examples of S-methylation. These glucosinolates break down during herbivory to produce several types of compounds including toxic thiols, which can then be converted by methylation to the volatile methyl esters such as methanethiol and methylthiocyanate (Fig. 11.1).[23]

Quaternary ammonium compounds are important as osmoprotectants in times of water deficiency and cold stress, as well as being constituents of membrane phospholipids. β-Alanine and phosphoethanolamine are each methylated three times on their nitrogens to produce the products β-alanine betaine and phosphocholine, respectively (Fig. 11.1).[24-27] An example of O-methylation and N-methylation occurring on the same molecule can be found in the indole alkaloid vindoline from *Catharanthus roseus* (Fig. 11.1).[28,29]

Types of Methyltransferases in Plants

The most widely used methyl donor for enzymatic methyl transfer is the cofactor S-adenosyl-L-methionine (SAM). The methyl moiety on the L-methionine is supplied by another known methyl donor, N^5-methyl tetrahydrofolate.[30] To date, numerous enzymes that perform SAM- dependent methylation reactions have been described in plants, and several reports attempting to sort out their evolutionary relationships have been published.[31-33]

It has now been recognized that the type of substrate being used is not a reliable prediction as to the provenance of the methyltransferase (MT) catalyzing the reaction. A large group of monophyletic O-methyltransferases (OMTs) with a molecular mass of approximately 40 kDa have been identified.[34,35] Enzymes in this group are often referred to as the small molecule OMT (SMOMT) family. Within this family, several subfamilies have been characterized, based on the degree of sequence similarity, which mainly, but not exclusively, correlates with the types of compounds upon which the enzymes act. For example, the enzymes responsible for the methylation of hydroxycinnamic acid derivatives are more similar to one another than they are to those involved in the methylation of hydroxyl groups of isoflavonoids and phenylpropenes, although there are exceptions, for example *Clarkia breweri* (iso)eugenol MT, which arose within the hyroxycinnamic acid OMT clade.[36] Another clade within this group consists of enzymes that O-methylate mostly isoflavones, but also some phenylpropenes and phenolics.

Recently, the crystal structure of one such SMOMT from alfalfa, isoflavone OMT (IOMT), was solved.[37] The likely reaction mechanism of this OMT, deduced from the crystal structure, involves the base-assisted deprotonation of the acceptor's hydroxyl group, followed by a nucleophilic attack by the subsequent phenolate anion on the reactive methyl group of SAM. Modeling of related SMOMTs using the crystal structure of IOMT has also proven useful in understanding the evolution of

this group of enzymes. Two SMOMTs from sweet basil that catalyze the formation of methyleugenol and methylchavicol - EOMT, and CVOMT, respectively - and that share 90% identity with one another were modeled using the crystallographic data from IOMT. It was found that swapping a single key amino acid from EOMT in the acceptor binding site with the corresponding amino acid from CVOMT (and vice versa) was sufficient to change the substrate specificities of the resulting mutant enzymes.[34]

A second group of OMTs consists of proteins with a molecular mass of 23-27 kDa whose known substrates are CoA esters of hydroxylated cinnamic acids (HCCoAs).[38-41] These enzymes, which usually share between 70-95% identity with one another, are also homologous to the N-terminal region of catechol OMTs from rat and human.[42] When compared to the plant SMOMTs, the HCCoA OMTs contain small stretches of amino acid residues common with SMOMTs that are believed to be involved in SAM binding, but overall there appears to be no statistically significant similarity between the two types of OMTs.[34]

A third group of OMTs are the protein carboxyl MTs, whose molecular mass is approximately 27 kDa.[43] These proteins are found in almost all organisms, and they serve to repair damaged proteins. In plants, they have been shown to be important during stress and in seed viability.[14,44-46] Their sequences are related to the animal glycine N-methyltransferases (NMTs) and the RNA/DNA MTs. Furthermore, the plant protein carboxyl MTs share no similarity to the other plant MTs involved in specialized metabolism. The enzyme that methylates a carboxyl functionality of Mg-protoporphyrin IX to produce a methylester constitutes a fourth type of plant OMT. This chloroplastic protein, whose mature form has a molecular mass of 31 kDa, is related to MTs with similar functions from photosynthetic bacteria, but is not closely related to any other plant OMTs.[6]

A recently discovered MT that catalyzes the N-methylation of phosphoethanolamine in the first committed step leading to the production of choline, a precursor of the osmoprotectant glycine betaine, appears to be a novel type of a plant MT.[47,48] The enzyme, PEAMT, appears to be a head-to-tail gene fusion of duplicated MTs of an SMOMT type gene. Interestingly, PEAMT functions as a monomeric protein, in contrast to the homodimeric SMOMTs. Another NMT, putrescine NMT, is most similar to spermidine synthase from mammals, and bears no sequence identity to other plant MTs beyond several residues critical for SAM binding.[42,49]

The only plant sulfur MTs reported in the literature are those that methylate the thiol-containing breakdown products of glucosinolates. These enzymes have a molecular mass in the range of 26-31 kDa, and they appear to be most similar to the halide MTs found in halophytic species of plants and can in fact utilize halide ions as substrates, although they do so with poor binding and slow turnover rates.[23,50,51] Neither the sulfur MTs nor the halide MTs show any sequence similarity to the

previously discussed plant MTs. For a more detailed review of some of the classes of MTs discussed above, see Ibrahim and Muzac.[42]

THE SABATH FAMILY OF MTS: KNOWN ENZYMES

Recently, a new group of MT enzymes that share sequence similarity to each other but have no significant sequence similarity to the MTs listed above have been identified in plants. The first two enzymes isolated and characterized from this family were SAM:salicylic acid carboxyl MT (SAMT) from flowers of *Clarkia breweri* and SAM:benzoic acid carboxyl MT (BAMT) from snapdragon flowers.[52-54] These two enzymes catalyze the formation of the volatile esters methylsalicylate and methylbenzoate, respectively. Both proteins function as homodimers with subunits of approximately 40 kDa. In addition, the NMTs, caffeine synthase (TCS1) from tea (and theobromine synthases (CTS1/2, aka CaMXMT) from coffee, were characterized and shown to be structurally related to SAMT and BAMT.[55-57] More recently, another enzyme belonging to this new class of MTs, this time from *Arabidopsis thaliana,* was shown to be SAM:jasmonic acid carboxyl MT (JMT) responsible for the formation of the volatile methyljasmonate.[58] To distinguish this group of MTs from those reported previously, we have designated this new class the SABATH family, based on three of the first five genes (**SA**MT, **BA**MT, and **Th**eobromine synthase) to be isolated and characterized.

Structural Features of the SABATH Family

All SABATH proteins characterized so far contain between 357 and 389 amino acids, and the molecular mass of the subunit ranges from 40-49 kDa. Size exclusion chromatography, performed with some of these enzymes, has revealed that the molecular mass of the native enzymes is roughly 80 kDa, indicating a dimeric structure. None of the SABATH proteins identified thus far have any obvious subcellular targeting signals. Cytosolic localization was experimentally confirmed for two of these enzymes, BAMT and CaMXMT.[57,59]

In addition to SAMT from *C. breweri*, SAMT genes have been isolated from two additional plant species, the tropical plant *Stephanotis floribunda* and the evening nightshade *Atropa Belladonna* (Genbank accession numbers AJ308570 and AB049752).[60] The SfSAMT and AbSAMT proteins share 61% identity with one another and are 55% identical to *C. breweri* SAMT. Parsimony and neighbor joining

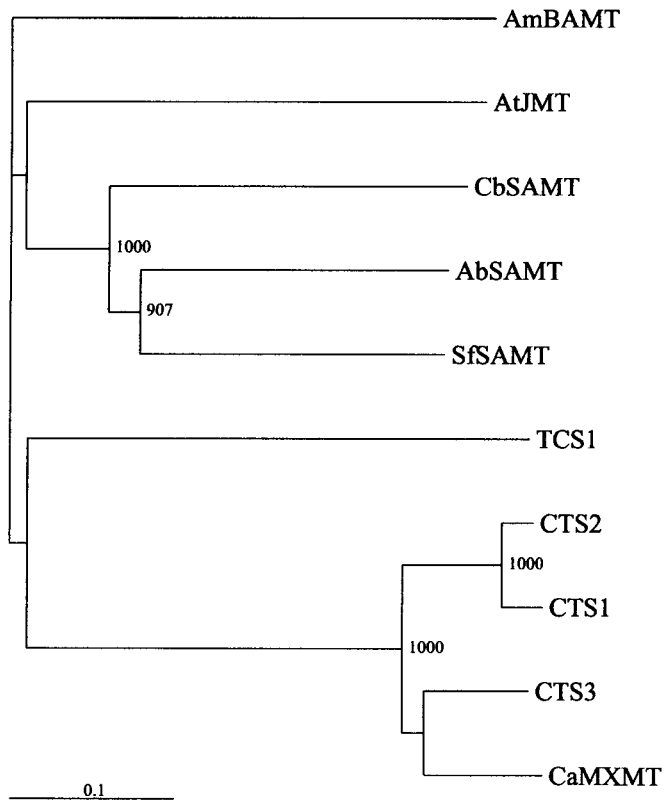


Figure 11.2: A phylogenetic tree consisting of SABATH MTs with known functions. The neighbor-joining tree was constructed using the aligned protein sequences of 10 SABATH MTs. Only statistically significant bootstrap values at the branch nodes are indicated. The resulting tree has three clades. The first contains only one protein, SAM:benzoic acid carboxyl MT (BAMT) from *Antirrhinum majus*. The second clade consists of SAM:jasmonic acid carboxyl MT (JMT) from *Arabidopsis thaliana* and SAM:salicylic acid carboxyl MT (SAMT) from the three species, *Clarkia breweri*, *Atropa belladonna*, and *Stephanotis floribunda*. The last clade contains the SABATH MTs involved in caffeine biosynthesis, caffeine synthase from *Camellia sinensis* (TCS1), and theobromine synthases (CTS1-3, and CaMXMT) from *Coffea arabica*. Branches are drawn to scale with the bar indicating 0.1 substitutions per site.

analyses indicate that the three SAMT proteins cluster together into their own clade, separate from the other two known SABATH carboxyl MTs, BAMT and JMT (Fig. 11.2). The SABATH enzymes that are involved in N-methylation of caffeine precursors form a distinct clade from the known carboxy MTs (Fig. 11.2). With the exception of the three SAMT sequences, which are at least 55% identical to each other, the other known SABATH proteins are only 40-45% identical to one another at the amino acid level.

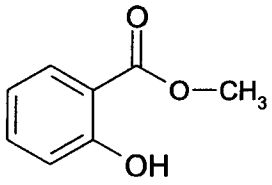
The crystal structure of *C. breweri* SAMT in a complex with salicylic acid and S-adenosylhomocysteine (SAH) has recently been solved to a resolution of 3Å (Zubieta, Koscheski, Ross, Pichersky, Noel, unpublished, see also Chapter 2, this volume). SAH was used instead of SAM because SAM is unstable. The SAMT crystal structure verifies that the SABATH MTs are structurally very different from any of the other groups of MTs previously characterized. For example, while SAMT is a homodimer like IOMT, the dimerization interface of SAMT involves only 7% of the available surface area of the dimerization region, while in IOMT as much as 30% is buried in the interface. In addition, each of the two substrate-binding sites of the IOMT homodimer utilizes amino acids from both monomers in forming the back wall of the site.[37] This is not the case in SAMT, where each of the two substrate-binding sites is made up from amino acids of a single monomer.

Amino acids contributing to the binding of SAM via several hydrogen bonds are similar between SAMT and the other crystallized SAM-dependent MTs such as IOMT, ChOMT, and COMT, suggesting either convergent evolution or modular evolution.[37,61] Unlike the situation in IOMT, there appears to be no catalytic residues required for methyl transfer in SAMT. Instead, the positioning of the nucleophilic carboxy anion of salicylic acid near the positively charged sulfur of SAM is sufficient to achieve methyl transfer.

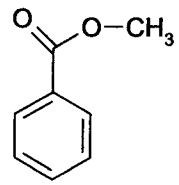
Specialized Compounds Made by SABATH Enzymes

The volatile ester methylsalicylate (Fig. 11.3) has several important biological functions in plant biology. It is a constituent of the scent of many different species of flowering plants.[7,62] In the California wildflower *Clarkia breweri*, it makes up about 5% of the total scent output of the flower and is thought to be an important attractant to pollinators.[63,64] The moth pollinator of *C. breweri*, *Hyles lineata,* has a strong electroantennogram (EAG) response to methylsalicylate.[65] In the tropical flower *Stephanotis floribunda*, methylsalicylate emission is regulated in a nocturnal fashion, with peak volatile production occurring in the early night.[60] Furthermore, methylsalicylate is emitted from the vegetative tissues of many plants that are damaged by predation or are infected by viral, bacterial, or fungal pathogens.[66-69] For example, tobacco plants infected by the tobacco mosaic virus emit methylsalicylate from their leaves.[70-72] Another common volatile present in the headspace of many flowering plants is methylbenzoate (Fig.

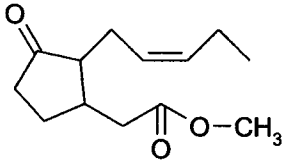
11.3). In snapdragon flowers, methylbenzoate is the most abundant floral volatile and is emitted in the upper and lower lobes of the petals.[54,73,74] The emission of this volatile is rhythmically regulated with the highest emission occurring during the day.[75]



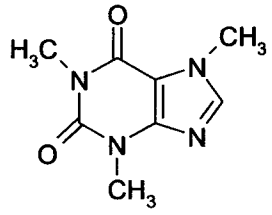
Methyl Salicylate



Methyl Benzoate



Methyl Jasmonate



Caffeine

Figure 11.3: Products of the biochemically characterized SABATH MTs.

Plants utilize methyljasmonate not only for floral scent but also as a cellular regulator that controls a myriad of plant functions.[76] The *cis* epimer (Fig. 11.3) is believed to be the most biologically active.[77] Like methylsalicylate, methyljasmonate and its precursor jasmonic acid have been implicated in the induction of defense-related genes in plants that are damaged by herbivores or challenged by infection.[78,79] Application of methyljasmonate either directly to the plant or in a volatilized spray can induce the expression of known pathogenesis-related proteins.[80-82]

Caffeine (Fig. 11.3), widely used by humans as a stimulatory drug, has so far been detected only in a few plant species. The biological roles of caffeine are believed to be in defense against herbivory or as an allelopathic response to potential competitors.[83] Caffeine is derived from the purine alkaloid xanthosine.[84] From xanthosine, three methylations are necessary to produce caffeine. First, xanthosine is methylated on N7 by 7-methylxanthosine synthase (MXS or 7NMT) to produce 7-methylxanthosine, which is enzymatically hydrolyzed to produce 7-methylxanthine and ribose.[85,86] The methylations of N1 and N3 of 7-methylxanthine to produce 1,3,7-trimethylxanthine (caffeine) occur in young leaves of tea, and the same enzyme, caffeine synthase, apparently catalyzes both reactions.[55] In coffee plants, caffeine is mainly found in the beans but also occurs in the leaves. Caffeine is stored in the vacuoles of coffee leaves as a complex with polyphenols such as chlorogenic acid.[87] In contrast to tea, coffee plants appear to have separate enzymes for each step of N-methylation.[57]

PHYLOGENETIC ANALYSIS OF THE SABATH FAMILY IN *Arabidopsis thaliana*

While SABATH MTs with important functions have been described in several plant species that are relatively little studied otherwise, the function of only a single SABATH gene from *Arabidopsis*, JMT, has so far been determined.[58] The recently completed genomic sequence of *Arabidopsis thaliana* now allows for an exhaustive search of related genes of any gene family. We undertook an *in silico* study to identify all the members of the SABATH gene family in *Arabidopsis*. Protein sequences obtained from the previously characterized SABATH genes were used to search the annotated genome sequences of *Arabidopsis* using the TBLASTN program.[88] The newly gathered protein sequences were used in further probing the genome in a reiterative approach. In order to analyze the protein sequences further, the original gene annotations performed by members of the *Arabidopsis* Genome Initiative (AGI) were compared with publicly available EST databases and adjusted for any discrepancies in translational start sites and location of intron/exon splice sites. In several cases, the original predictions of either start/stop or splice sites based on the information gathered from members of the AGI were found to be incorrect. In addition, the EUGENE gene prediction software was also used to aid in the identification of protein sequences.[89]

Our analysis revealed the presence of 24 SABATH genes and one pseudogene in the genome of *A. thaliana* (Table 11.1). Alignment of the *Arabidopsis* SABATH proteins as well as non-*Arabidopsis* SABATH proteins with known function reveals that protein identity among family members ranges from 23% to 95%. Neighbor-joining analysis gives a tree with three main branches, designated here as Groups I, II, and III. The AtSABATH proteins all fall into Groups

I and III, with the exception of JMT (Fig. 11.4). JMT, the only characterized protein from *Arabidopsis*, does not seem to be closely related to any of the other AtSABATH sequences but instead joins the SAMT proteins from differing species in a separate clade, Group II, that also includes the known NMTs involved in caffeine biosynthesis. BAMT from snapdragon does not fall within the SAMT clade but instead is more similar to the AtSABATH proteins in Group III. It is also interesting that the genes that are in the 3- and 4-gene clusters within the *Arabidopsis* genome (see below) encode for proteins that, with one exception, all belong to Group I.

INTRONS IN AtSABATH GENES

There are a total of 5 positions within the AtSABATH genes in which introns may occur. However, no AtSABATH gene has more than 4 introns, and nearly all the AtSABATH genes contain 2 (15/24) or 3 (7/24) introns (Table 11.1, Fig. 11.5). In all cases in which an intron occurs in the same position in more than one gene, the phase of the intron is conserved. To aid in the identifications of intron positions, we numbered them in the order in which they appear from the 5' end of the genes (Table 11.1, Fig. 11.5). Four genes, At5g04370, At5g04380, At3g11480, and At5g37990, have an N-terminal region that is longer than the predicted proteins. This extension ranges from 17 to 45 amino acids. The sequence of one of these genes, At3g11480, has been confirmed by performing 5' RACE (unpubl. data). Of those four sequences, At5g04370, At3g11480, and At5g37990 have an intron after the codon encoding an arginine residue at position 13 relative to At3g11480 (intron 1). These three genes cluster in Group III of the phylogenetic tree, whereas the fourth, At5g37970, is placed within a clade in Group I (Fig. 11.4). At5g37970 is found in a cluster of several other Group I SABATH genes on chromosome V (see below, and Figs. 11.6,7).

The second position at which an intron occurs is conserved by all but 2 sequences. This position occurs immediately after the codon encoding the amino acid asparagine at position 25 relative to JMT, which is a conserved codon in all genes containing this intron. The two sequences that do not have an intron at this location are At5g56300 and At4g26420. These two genes pair together in Group I (Fig. 11.4), and their proteins are 57% identical to one another.

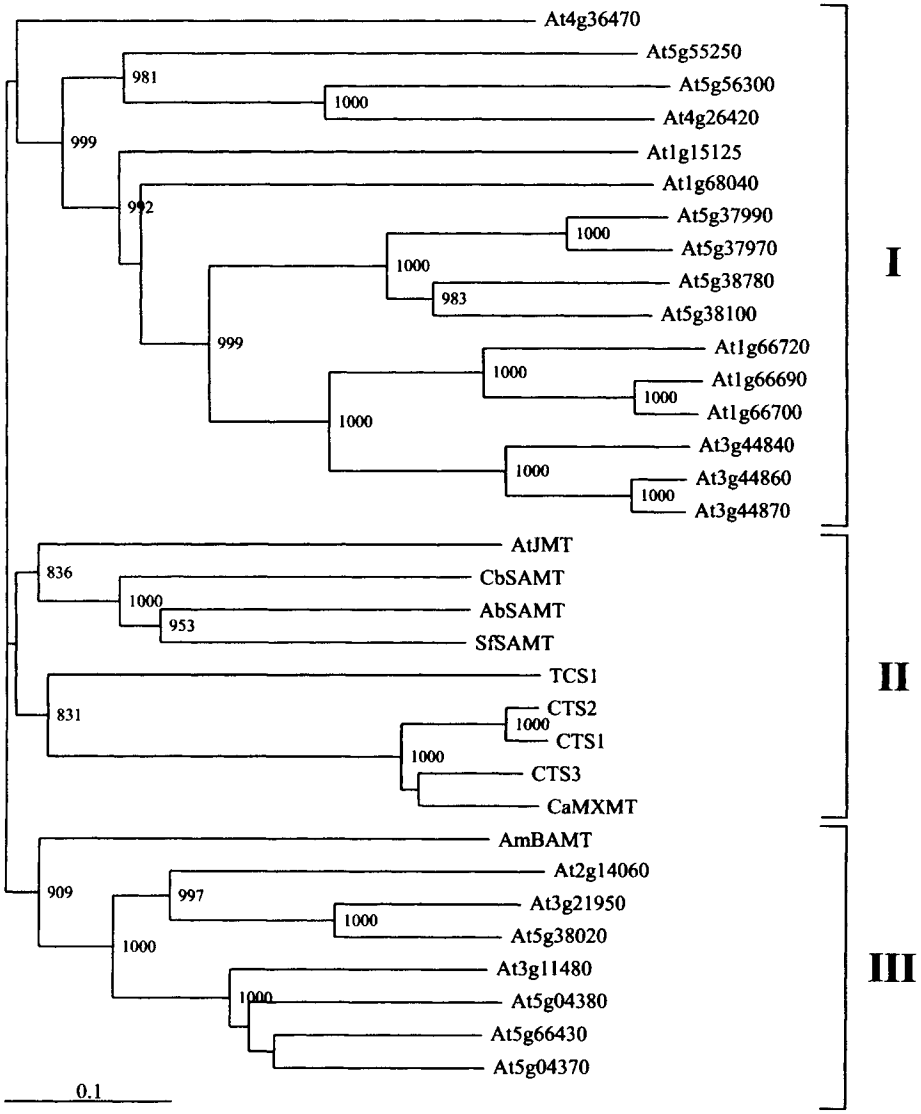

At4g36470
At5g55250
981
At5g56300
1000
At4g26420
999
At1g15125
At1g68040
992
At5g37990
1000
At5g37970
1000
At5g38780
983
At5g38100
999
At1g66720
1000
At1g66690
1000
At1g66700
1000
At3g44840
1000
At3g44860
1000
At3g44870
I
AtJMT
836
CbSAMT
1000
AbSAMT
953
SfSAMT
TCS1
831
CTS2
1000
CTS1
1000
CTS3
CaMXMT
II
AmBAMT
909
At2g14060
997
At3g21950
1000
At5g38020
1000
At3g11480
1000
At5g04380
At5g66430
At5g04370
III
0.1

Figure 11.4: Phylogenetic tree of the SABATH family that includes all the SABATH proteins from *Arabidopsis thaliana* and SABATH proteins of known function from other species. The neighbor-joining analysis was performed using the 24 AtSABATH protein sequences and the sequences of the 10 previously characterized SABATH proteins. The resulting trichotomous tree is shown here with statistically significant bootstrap values reported at the nodes. The SABATH proteins fall into three distinct groups. Group I consists entirely of AtSABATH sequences, including the proteins encoded by genes found in three gene clusters, one each on chromosomes I, II, and V. Group II consists exclusively of the SABATH proteins with known function with the exception of BAMT. BAMT and the rest of the AtSABATH proteins, including those encoded by a 2-gene cluster on chromosome V, make up Group III. Branches are drawn to scale with the bar indicating 0.1 substitutions per site.

At1g15125 is the only AtSABATH gene that contains intron 3, and this intron is the only example of the gain of an intron that is not shared by at least three AtSABATH genes. The splice site of this intron occurs after the glycine codon 138 (using the JMT open reading frame for the codon numbering), and was confirmed by both EST data and RT-PCR (unpublished). At1g15125 contains a total of 3 introns, two of which are also found in At1g68040, the gene that encodes the protein most similar to the one encoded by At1g15125 (35% identity).

An intron in the fourth intron position occurs in nine of the AtSABATH genes, including JMT. The splice site occurs after codon 167 (JMT numbering), a conserved glutamine or lysine codon. Both Groups I and III have clusters of 4 genes containing this intron. A loss of the intron basal to these clades can explain its absence in the other sequences (Fig. 11.6). Lastly, all AtSABATH genes contain intron 5, at codon 263 relative to JMT. Although all AtSABATH genes contain this intron, the corresponding amino acid in this position is not conserved.

Table 11.1: The *Arabidopsis thaliana* SABATH gene family

Gene[a]	Strand on chromosome	Start Codon	Stop Codon	Number of Introns	Protein Length (AA)
At1g15125[b]	+	5204621	5205908	3 (2,3,5)[d]	351
At1g19640 (JMT)	-	6791722	6788871	3 (2,4,5)	389
At1g66690	-	24522762	24521466	2 (2,5)	353
At1g66700	-	24526602	24525372	2 (2,5)	353
At1g66720	+	24533148	24534392	2 (2,5)	352
At1g68040	+	25154788	25157136	2 (2,5)	363
At2g14060	-	5864319	5863066	2 (2,5)	368
At3g11480[b]	+	3614552	3617145	4 (1,2,4,5)	379
At3g21950[b]	+	7734385	7736190	2 (2,5)	368
At3g44840	-	16382719	16381459	2 (2,5)	348
At3g44860	+	16388649	16389899	2 (2,5)	348
At3g44870	+	16391237	16392565	2 (2,5)	382
At4g26420[c]	-	12316983	12315366	2 (4,5)	383
At4g36470	-	16180964	16179617	3 (2,4,5)	362
At5g04370	+	1232026	1233887	4 (1,2,4,5)	396
At5g04380	+	1234882	1236316	3 (2,4,5)	385
At5g37970	-	14838594	14836999	3 (1,2,5)	412
At5g37990	-	14849079	14847777	2 (2,5)	362
At5g38020	-	14881522	14879863	2 (2,5)	368
At5g38100	-	14915078	14912915	2 (2,5)	359
At5g38780	+	15243995	15245373	2 (2,5)	361
At5g55250	-	22124768	22121456	3 (2,4,5)	386
At5g56300	+	22513498	22515390	2 (4,5)	380
At5g66430	-	26240704	26239316	3 (2,4,5)	363
pseudo-1 (At1g66710)	-	24532172	24530010	-	-

[a]Gene names are designated by the AGI naming scheme, in which the chromosome number is given after the "At" designation, followed by "g" and a five-digit number.

[b]EST or mRNA data differ from the AGI annotations at the site of the start codon for these genes.

[c]The AGI annotation for At4g26420 contains extra coding sequence at the C-terminal end that is not in agreement with our predictions based on protein sequence alignments and EUGENE gene prediction software.

[d]Numbers in parentheses refer to the positions of the introns as indicated in Fig.11.5.

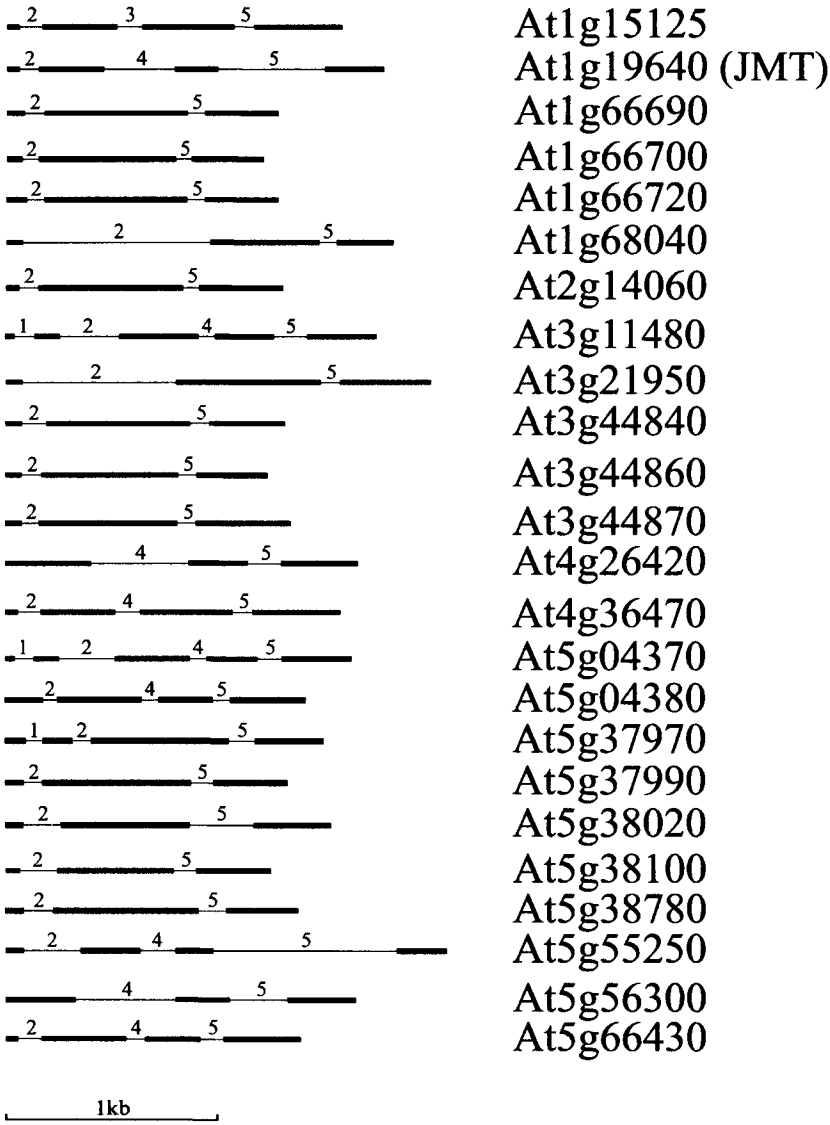


Figure 11.5: The distribution of introns in members of the AtSABATH gene family. AtSABATH genes are drawn to scale from the ATG start site to the stop codon of the predicted open reading frame. Heavy and thin lines represent the predicted exons and introns, respectively. Numbers above each line represents one of the five possible intron positions found in the AtSABATH gene sequences.

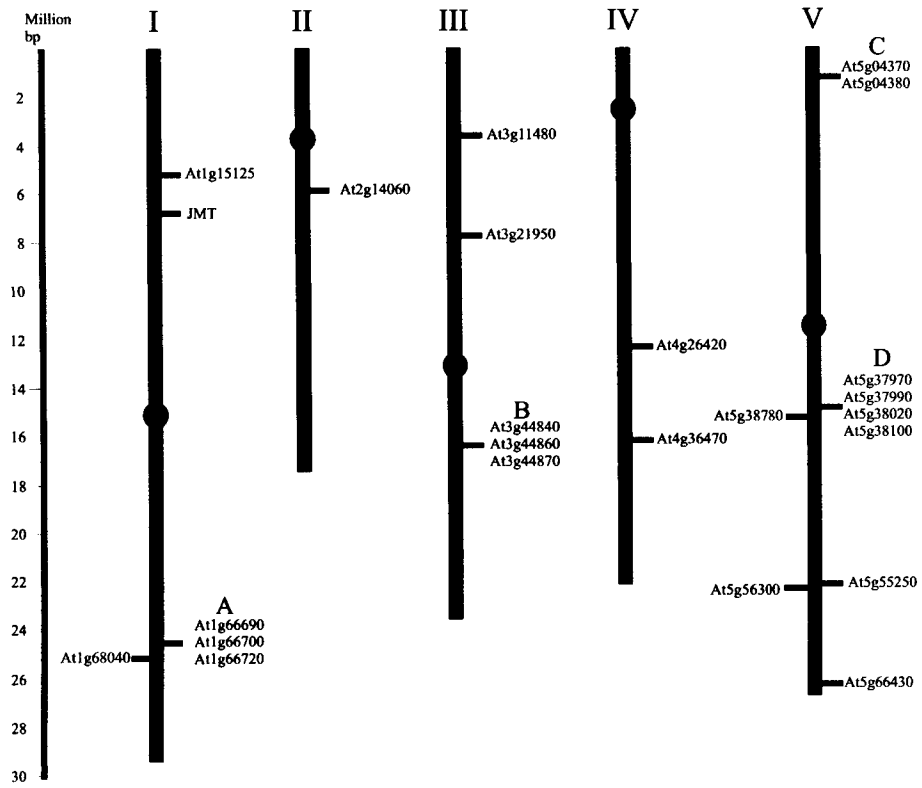


Figure 11.6: Chromosome localization of the AtSABATH genes. Gene names are annotated using the nomenclature proposed by the AGI. Circles denote the centromeric regions of each chromosome. The four clusters of AtSABATH genes are (A) chromosome I: At1g66690, At1g66700, and At1g66720 (B) chromosome III: At3g44840, At3g44860, and At3g44870 (C) chromosome V: At5g04370 and At5g04380 (D) chromosome V: At5g37970, At5g37990, At5g38020, and At5g38100.

GENOMIC ANALYSIS OF THE SABATH GENE FAMILY IN *Arabidopsis thaliana*

Linkage of AtSABATH Genes to Each Other

The AtSABATH genes are scattered throughout the five chromosomes, with half of the genes clustering in groups of two to four (Figs. 11.6,7). Chromosome V harbors the largest number of SABATH genes with a total of 10, including a cluster of two genes and a cluster of four genes. Chromosomes I and III contain six and five SABATH genes, respectively, with each chromosome harboring a cluster of three genes. Two genes are present on chromosome IV, and the remaining SABATH gene is found on chromosome II.

Analysis of the identity of neighboring genes may help to understand better the function of SABATH genes in two important ways. First, the presence of a nearby SABATH gene may suggest that gene duplication and divergence have occurred, and that the two genes encode enzymes with similar (or identical) functions. Second, both structurally related and structurally unrelated neighboring genes may be involved in the same pathway, and thus identifying the substrate of the enzyme encoded by one gene may help in identifying the substrate for the other. Indeed, it has been hypothesized, and in some cases demonstrated, that genes involved in the same pathway of secondary metabolism in plants may be found in close proximity to each other. For example, several P450 oxidoreductase genes as well as indole-3-glycerol phosphate lyase (IGL) involved in the biosynthesis of DIBOA are tandemly linked in maize.[90,91] In *Arabidopsis*, several clusters of acyltransferase genes as well as genes encoding terpene synthases and P450 enzymes have been documented.[92,93]

With these considerations in mind, the two tandemly linked SABATH genes (At1g66690 and At1g66700) on chromosome I, which encode proteins that are 95% identical on the amino acid level, are probably the result of a recent duplication event (Fig. 11.7A). The third SABATH gene in this cluster, At1g66720, is in the opposite orientation and encodes a protein that is approximately 73% identical to the proteins encoded by the two neighboring SABATH genes. The intervening gene in this group, At1g66710, appears to be a SABATH pseudogene based on the observations that it contains multiple stop codons within the putative open reading frame.

The 3-gene cluster present on chromosome III is similar to the cluster of genes on chromosome I, with the exception that no pseudogene is present (Fig. 11.7B). The two tandemly linked genes, At3g44860 and At3g44870, encode proteins that are more similar to each other (87%) than to the oppositely oriented gene, At3g44840 (71%). The 2-gene cluster on chromosome V also consists of tandemly linked genes At5g04370 and At5g04380 whose proteins are 68% identical to one another (Fig. 11.7C). However, the phylogenetic analysis shows that

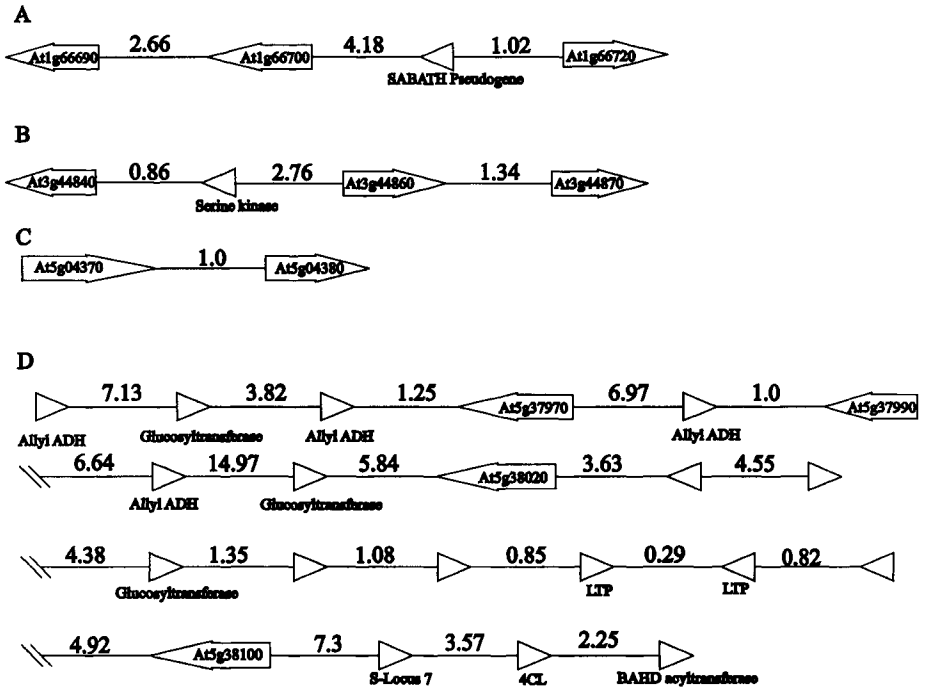


Figure 11.7. Physical map of the regions containing AtSABATH gene duplications. AtSABATH genes are represented here as arrows with their AGI designation, and triangles denote other genes in the region. The orientation of each gene is represented by an arrow (pointing in the direction of transcription). In cases where an encoded protein has high similarity to a previously characterized protein, a putative designation is given underneath the triangle. Thin lines with distances (in kb) indicate the physical separation between identified open reading frames. Hatched bars represent the continuation of the physical region from the above line. (A) The three gene cluster of AtSABATH genes on chromosome I. The intervening gene between At1g66700 and At1g66720 is the only recognized AtSABATH pseudogene. This pseudogene contains multiple stop codons within its putative open reading frame. (B) The 3-gene cluster of AtSABATH genes on chromosome III. The intervening gene between At3g44840 and At3g44860 has been identified as encoding a putative serine kinase. (C) The 2-gene cluster on the upper arm of chromosome V. (D) The 4-gene cluster on chromosome V. Predicted genes in this region include several allyl alcohol dehydrogenase-like genes, several glucosyltransferase genes, two lipid transfer-like genes, an S-locus7-like gene, a 4-coumarate CoA ligase-like gene, and a BAHD acyltransferase sequence.

another gene on chromosome V, At5g66430, is more similar to At5g04370 (72% identity on the protein level) than the two tandemly linked genes are with one another (Fig. 11.4).

The 4-gene cluster of SABATH genes on chromosome V (Fig. 11.7D) does not follow the same pattern of the aforementioned SABATH gene clusters. None of the SABATH genes in this region are tandemly linked to one another, and they do not belong to the same branch of the phylogenetic tree. The first two SABATH genes in this region, At5g37970 and At5g37990, belong to Group I and encode proteins that share 87% identity with one another. However, they only share 32% identity with the protein encoded by the next SABATH gene, At5g38020, which belongs to Group III. The most closely related gene to At5g38020 is At3g21950 on chromosome III, whose proteins share 67% identity. The last SABATH gene in this cluster, At5g38100, belongs to Group I, and its protein is 66% and 67% identical to the proteins encoded by At5g37970 and At5g37990, respectively.

Linkage of AtSABATH Genes to Other Genes of Potential Interest

Genes that are likely to encode enzymes for specialized metabolism are present in the vicinity of several AtSABATH genes. For example, a putative P450 oxidoreductase enzyme is located 24 kb upstream of the AtSABATH gene At4g36470. However, since the family of P450s in *Arabidopsis* comprises 0.6% of the genome (273 genes), it is possible that this proximity is caused by chance alone. An association of potential significance may be the one between the SABATH gene At2g14060 (on chromosome II) and an RPP1-like disease resistance gene. This latter gene encodes a protein similar to a nucleotide binding site–leucine-rich repeat protein that is known to be involved in the resistance of *Arabidopsis* to several strains of *Peronospora parasitica* (downy mildew).[94] In close proximity to these two genes resides a sequence encoding a P450 enzyme in the CYP705 subfamily. There is currently no documented function for this class of P450s, although this subgroup in particular is noted for being the result of repeated gene duplications.[95] Therefore, it is possible that the genes in this region participate in some way in the defense towards pathogenic challenge.

The *Brassica* S-locus region contains a SABATH gene that is expressed in the stigma during the latter stage of flower development just before the flower opens.[96] This expression pattern is similar to the pattern of expression of the S-locus genes *SLG* and *SRK* that are essential for the self-incompatibility response. However, further experiments revealed that this Brassica SABATH gene is not required for the specificity of the self-incompatibility reaction.[97,98] Interestingly, the orthologous region to the *Brassica* S-locus in *Arabidopsis* (on chromosome I) does not contain a SABATH gene. However, the *Arabidopsis* SABATH gene At1g15125 is within 17 kb of this region, but it is only 35% identical on the amino acid level to the Brassica S-locus SABATH protein.

Within the AtSABATH gene clusters of 4 genes on chromosome V, several sequences with similarity to genes potentially involved in flavonoid biosynthesis are present (Fig. 11.7D). The first encodes an enzyme similar to a tobacco NADP-dependent ally alcohol dehydrogenase that can act on the S stereoisomers of secondary allylic alcohols such as the monoterpenoid alcohols carveol and verbenol.[99] The next gene, which is in a head-to-tail orientation, encodes an anthocyanin glucosyl transferase-like protein and is followed by another allyl ADH-like gene. Following this gene in a head-to-head orientation is the first SABATH gene At537970. The next gene is another allyl ADH-like gene, followed by the second SABATH gene (At5g37990), again in a head to head orientation. A similar pattern of allyl ADH and glucosyl transferase genes preceding a SABATH gene is repeated once more with the SABATH gene At5g38020. The final gene in the cluster, At5g38100, is separated from the other SABATH genes by eight intervening genes, including one that encodes a putative transmembrane protein, another encoding a glucosyltransferase, and two genes encoding lipid transfer proteins. Downstream of At5g38100 is a gene with similarity to an anti-silencing factor found in the S-locus of *Brassica napus*. Finally, two more downstream genes in this region encode a 4-coumarate CoA ligase-like (4CL) and a BAHD acyltransferase protein.

AtSABATH GENE EXPRESSION ANALYSIS

We have recently begun an investigation of the expression of AtSABATH genes in different tissues and under different environmental conditions. The first step involves an *in silico* search of the publicly available EST databases. From this search, we have found several AtSABATH genes that are expressed in developing seeds and siliques, including At5g56300, At4g26420, and At1g19640.[100,101] In addition, At1g15125, At3g44860, and At1g68040 have been found in the above-ground organs of two- to six-week old plants. One gene, At3g44860, was identified in a cDNA library enriched for transcripts induced with 160 mM NaCl.[102] Finally, four genes- At1g66690, At3g44840, At3g44860, and At3g44870 - are expressed in liquid-cultured seedlings. The absence of other AtSABATH ESTs in the publicly available databases can be inferred to mean that the relevant conditions in which these genes may be expressed have not yet been used, or that some of these genes are expressed only at very low levels.

The second approach we have used to examine the expression of members of the AtSABATH gene family is by RT-PCR methodology with gene-specific primers designed to span either the entire open reading frame or several exons. The RNA

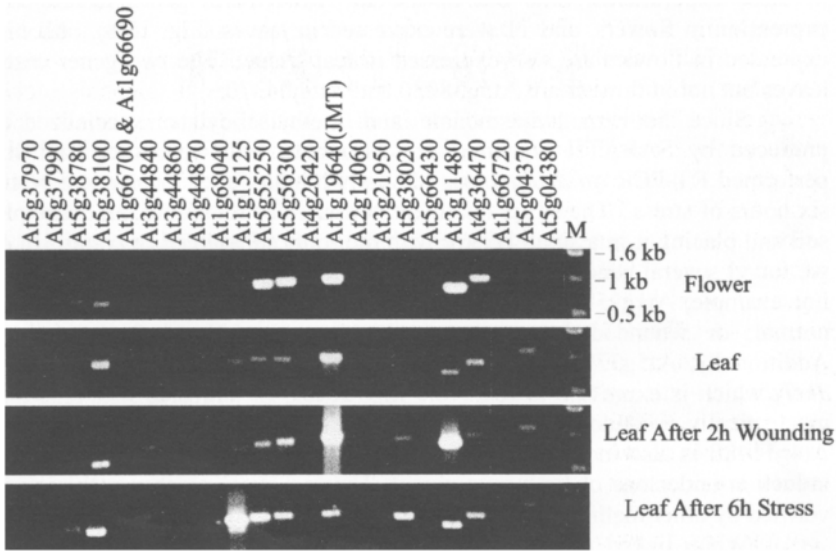


Figure 11.8. Expression of AtSABATH genes. Flowers and leaves were collected from six-week old flowering plants as well as from leaves two hours after mechanical wounding and from leaves 6 hours after stress treatment. Total RNA was extracted and used for RT-PCR analysis. A pair of primers was designed that recognized At1g66690 and At1g66700 equally well since these two genes are 95% identical to one another (both genes could be amplified from genomic DNA with the pair of primers designed against At1g66700). Overall, the expression of 11 genes was detected in leaves, 9 in flowers, 11 in leaves 2 hours after mechanical wounding, and 12 in leaves 6 hours after stress treatment. Twelve genes (At1g66690, At1g66700, At1g66720, At2g14060, At3g21950, At3g44860, At3g44870, At4g26420, At5g04380, At5g37990, At5g66430) showed no expression in any of the four tissues. The lane labeled M contains DNA size markers.

used in these experiments was treated with DNase to remove DNA contamination. In these experiments, nine out of the 24 AtSABATH genes examined showed expression in flowers, and 11 were expressed in leaves (Fig. 11.8). All nine genes expressed in flowers are also expressed in leaf tissue. The two genes expressed in leaves but not in flowers are At5g38020 and At5g04370.

Since both methyljasmonate and methylsalicylate, specialized products produced by SABATH enzymes, are associated with wounding and stress, we performed RT-PCR on *Arabidopsis* leaves two hours after mechanical wounding or six hours of stress. The stress treatment consisted of removing the entire plant from soil and placing it in a small beaker with the roots submerged in water. In doing so, we found several genes that are expressed differentially under these two treatments. For example, At1g15125 is normally expressed at relatively low levels in flowers, normal, or wounded leaves, yet is highly up regulated by stress treatment. Additionally, At5g37970 is expressed only in leaves after 6h stress. In contrast, *JMT*, which is expressed at relatively high levels in untreated tissue, is induced in mechanically wounded leaves but is down regulated by stress treatment. Gene At4g11480 is also induced in wounded leaves. Other genes show lower levels of induction under one or both treatments. These expression characteristics are being verified by other methods, such as RNA blotting and Promoter-GUS fusion analysis.

When linked to volatile analysis or other types of metabolic profiling of *Arabidopsis* plants subjected to different treatments, the observed changes in gene expression could prove useful in predicting functions of individual genes. For example, herbivory by the caterpillar *Pieris rapae* on *Arabidopsis* plants causes the emission of methylsalicylate.[103] Thus, the examination of up-regulated genes of the AtSABATH family under caterpillar damage could provide good candidates for the identification of the *Arabidopsis* SAMT gene. Candidate genes can then be cloned into bacterial expression vectors, and the resulting proteins tested for their kinetic parameters (K_m and K_{cat} values, for example) with the hypothesized substrates, to determine if these compounds are likely to serve as substrates under physiological conditions.[52,104] Additional information concerning the biological functions of candidate genes may come from mutant plant lines and transgenic plants overexpressing such genes.

SUMMARY

SABATH MTs are found only in the plant kingdom, and they also do not display significant sequence similarity to other known plant MTs. However, they share several physical characteristics with some other families of plant MTs, such as forming homodimers and sharing similar SAM binding residues. The SABATH protein family appears to be unique among plant MT families in containing enzymes that transfer the methyl group to an oxygen while other members of the family

transfer the methyl group to a nitrogen; previously described plant MT families contain members that appear to modify only one type of functionality.

Although 24 SABATH gene family members have been found in *Arabidopsis*, the protein encoded by only one of them has been biochemically characterized. Further characterization of SABATH genes from *Arabidopsis* and other species of plants should help to provide a better understanding of the range and types of functional groups that this family of enzymes can modify by methylation. In addition, modeling of the active sites of *Arabidopsis* SABATH proteins with unknown function, using the crystal data of *Clarkia breweri* SAMT, may help in the identification of potential substrates, and may also assist in understanding the evolution of function in this gene family. The addition of sequence and linkgage data from other species such as *Arabidopsis lyrata, Medicago truncatula,* and *Oryza sativa* may also aid in our understanding of both the phylogenetic relationships among SABATH genes and their functions.

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Chapter Twelve

XOCHIPILLI UPDATED, TERPENES FROM MEXICAN PLANTS

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This chapter is dedicated to Herrn Professor Dr. Klaus Hahlbrock (Max-Planck Institute for Plant Breeding Research) for his many significant contributions and continuous support in the advancement of knowledge of plant secondary metabolism.

INTRODUCTION

Terpenes are among the most abundant plant chemical compounds, with many roles in plant physiology. Despite complicated structures, their general biosynthesis is relatively simple and flexible. Among their many roles, they are involved in chemical defense, (*e.g.*, phytoalexins), and growth regulation (*e.g.*, gibberellins). Some target specific processes in animal physiology and have been used for a long time for human health needs. Their properties have stimulated the search for more terpene-containing plants as medicines. The resources of a region may depend on the amount and variety of plants. México is one of the richest countries in medicinal plant varieties. There is old and detailed knowledge of plant properties. Ancient cultures such as the Maya, Aztec, and Zapotec had the skills and experience to use plants as treatments for several illnesses, as well as in important cultural events including religious ceremonies. The owners of this knowledge were highly respected and provided the advancement of their practices into the development of sophisticated remedies. The Spanish conquest changed these concepts and interrupted their development and use. The conflict between the two cultures turned the sacred and respected role of healers and midwives into demoniac and pagan practices subject to the Inquisition. Fortunately, however, the knowledge of plant uses and properties was preserved, mainly by word of mouth, and some few graphic documents were preserved. A detailed description of Mexican medicinal plants and their uses is illustrated in the Badianus Codex, an Aztec herbal of 1552. This manuscript, written by an Indian physician in Náhuatl and translated into Latin by another Indian, provides a picture of Aztec medicine at the time of the conquest. As an herbal booklet, it deals with the pharmacological treatment of diseases.[1] Because it is illustrated, it is a valuable source of Aztec lexicography since the Aztec names were used. It has remedies for common indispositions such as foot problems, and outlines treatments for ulcers in the soles of feet, eruptions, swellings, and remedies for foot parasites like chigoes (jigger flea).

These traditions were mixed with Catholicism, especially Spanish uses and habits that resulted in a modified Aztec culture. As an example, the Badianus Codex describes a remedy for "black blood," translating the Aztec term for melancholy. Badianus used the words "black blood", a common term in sixteenth-century Europe, but the Aztecs considered melancholy not a visitation of an evil spirit but a definite physical disorder.

In this chapter, we summarize the current knowledge of interesting terpenes found in Mexican medicinal plants, with special emphasis on the zoapatle (*Montanoa tomentosa*), a plant originally called "cihuapahtli." This means woman's medicine, and it was used to assist women during difficult labor because of its specific uterus contraction effect caused by the diterpene active substances. Finally, some aspects of the current use of medicinal plants in different cultural environments will be discussed.

THE SEARCH FOR PAST KNOWLEDGE

"Tzintic" (It Began, It Originated, in Náhuatl)

"Xochipilli" is the Prince of Flowers of the Mexica or Aztecs of Mesoamerica. The statue was unearthed at Tlalmanalco on Mt. Popocatépetl and is dated approximately 1450 A.D. The god's body is adorned with carved flowers including "coaxihuitl" or the morning glory *Turbina corymbosa* (L.) Raf., source of "ololiuhqui" seeds, "quauhyetl" or the tobacco *Nicotiana tabacum* L., and other visonary plants, including rosettes of the sectioned caps of the mushrooms called "teonanacatl" (flower mushrooms) in Náhuatl, probably *Psilocybe aztecorum*. The face of "Xochipilli" is contorted as if seeing visions in ecstasy, and the head is tilted as if hearing voices. He is absorbed in "temicxoch", the flowery dream as the Náhuatl say in describing the awesome experience that follows the ingestion of an entheogen (http://mathrisc1.lunet.edu/~snow/xochi.html).

"Xochipilli" represents the Aztecs' knowledge of the effects of plants on humans. The search for medicinal plant uses by ancient cultures is beset with language problems. The translation from Náhuatl to Spanish was the first obstacle to be overcome. For example, when conquerors asked for help for their injured feet, the recipe they got was as follows: For injured feet, grind together these herbs: "tlalhecapahtli" (earth wind medicine), "coyoxiuitl" (rose colored bell plant), "yztauhyatl" (salty water plant), "tepechian" (mountain chia), "achilli" (flexible, reddish water plant), "xiuehcapahtli" (plant wind medicine), "quauhyyauhtli" (wild incense), "quetzalxoxouhcaphtli" (precious blue medicine), "tzotzotlani" (glistening plant), the flowers of "cacauaxochitl" (cacao flower), "piltzintecouhxochitl" (noble lord flower), and foliage of "hecapahtli" (wind medicine) and "ytzcuinpahtli" (dog medicine), the stone "tlahcalhuatzin" (bezoar stone of huatzin,a native bird), "eztetl" (bloodstone--a type of jasper) and "tetlahuitl" (red ochre stone), pale-colored earth. Put some in water in a little tub over embers or a fire to heat, and when the liquid has become hot, put the feet into the tub. Some part of it is to be inspissated by fire, and is to be applied to the feet. So that it will not run off, the feet are to be wrapped in a cloth. Next day the unguent "xochiocotzotl" (flower pine resin) and white incense are to be thrown on a fire so that the feet may become healthy from the odor and heat. Additionally, the seed of the herb called "xexihuitl" is to be ground, and when it has been pulverized in hot water, put on the feet. Finally, apply the herb "tolohuaxiuitl" (*Datura* plant) and briars ground in hot water.[1]

Plant names seemed difficult to remember, but they had specific rules. As noticed in the previous recipe, the classification of a plant was usually by one word composed of several phonemes or the smallest sound units which, in terms of the phonetic sequences of sound, controls meaning. Indeed, the simplest classification was by using two phonemes, quite similar to the current classification by genus and

species. The "pahtli, patli" (medicine, remedy, potion) suffix in any word was the general term to indicate a plant source used to treat diseases. So, several genera were classified as medicinal. In this way, "cihuapahtli" ("cihua" = women, "pahtli" = medicine or woman's medicine) indicated specific plant(s) for women's diseases. So, the Mexica "titici" (medicine man) developed one of the best classifications of medicinal plants at the time. Among the plant Náhuatl names, there were plant families, such as the sour fruits or "xocotl", sweet fruits or "tzapotl", and so on. They also had genus and species names, *e.g.*, "Cochitzapotl" (sweet fruit inducing sleep) currently *Casimiroa edulis*, a fruit used to induce sleep, also having substances that lower blood pressure, a "nutraceutical" at present.[2]

Another linguistic problem was caused by the healers and illnesses themselves. Among healers, there were several specialists, and illnesses were named according to their resulting effects. The first formal review conducted in México of these names and their interpretation was done through a program managed by the Mexican Institute for Social Security (Instituto Mexicano del Seguro Social, IMSS) aimed at a General Coordination of a National Plan for Depressed Zones and Marginal Groups (Coordinación General del Plan Nacional de Zonas Deprimidas y Grupos Marginados, COPLAMAR). The IMSS-COPLAMAR Program was performed between 1982-1987. After completing an initial program to understand the traditional medicine of Chiapas State, a second phase on a national scale was carried out.[3]

The result of the second phase showed that all across the country and in each Mexican State, there were more Traditional Therapists (TT) than formal Medical Doctors (MD) belonging to IMSS, an average of 4 TT for 1 MD in 1984. Different titles were used for different therapists such as "tepatiquez" (healers) and "tetlacachihuiliani" (one who brings about birth, or midwife). Spanish names were and are still in use, such as "sobadores" (people giving massage), "hueseros" (experts in skeleton-muscular system), "chupadores" (healers that suck out an illness), and others. In 1984, the IMSS-COPLAMAR program identified the names used for the traditional therapists around the country. These are shown in Table 12.1. This information provides a good example of the importance of traditional medicine in México.

The same program compiled the used names for the most common illness and their respective nosology, which is a systematic arrangement or classification of diseases (Table 12.2). The most interesting part of the program was the compilation of the plants used for illness, as well as the procedures to prepare the remedies. Because many plants were used repeatedly in several regions, the total number of plants was 5,773. After the phonetic grouping and the selection of synonyms, only 1,950 names were considered significant. From these plants, only those used in at least 18% of all investigated places were considered significant. Finally, just 104 plants were found widely distributed according to the program research.[3]

Table 12.1: Old and modern names used to identify Mexican traditional therapists according to their area of expertise. Program IMSS-COPLAMAR 1984.

OLD NAME	SPECIALTY	MODERN NAME
Curandera (Healer)		Boticaria (Apothecary)
Hierbera (Herbalist)	PARTERA	Médica (Woman physician)
Sobadora (Masseuse)	(Midwife)	Pastillera (Tablet's provider)
Espiritualista (Spiritualist)		Empírica (Empiric)
Pulsadora (Women who feel the pulse)		Capacitada (Qualified)
Adivino (Guesser)		Huesólogo (Bonesetter)
Brujo (Sorcerer)	CURANDERO	Inyectador (Injector man)
Ensalmador (Bonesetter)	(Healer)	Recetador (Medicine's prescriber)
Huesero (Bonesetter)		Cancerólogo (Cancer specialist)
Exorcista (Exorcist)	HUESERO	Homeopático (Homoeopathic)
Hechicero (Bewitcher)	(Bonesetter)	Masajista (Masseur)
Hierbero (Herbalist)		Huesólogo (Bonesetter)
Rezandero (Prayer)		Empírico (Empiric)
Sobatripa (Bowelmasseur)	HIERBERO	Farmacéutico (Pharmacist)
Brujo (Sorcerer)	(Herbalist)	Doctor (Physician)
Granicero (Hailstorm producer)		Paramédico (Paramedic)

Modified from Ref. 3

Table 12.2: Classification of the ten more common diseases of Mexican traditional medicine. Program IMSS-COPLAMAR 1984.

NOSOLOGY	INVOLVED APPARATUS OR SYSTEM (% REFERENCE) OBSERVATIONS
MAL DE OJO (Evil eye)	Digestive (51.18%) Affecting children under the age of three. Produced by a particular look to the child by a person having a "strong or hard look". The symptoms are like gastroenteritis most probably infectious.
EMPACHO (Indigestion) Empacho seco (dry indigestion), empacho pegado (stick indigestion), trazo de empacho (outline's indigestion), empacho ligero (light indigestion), empacho fuerte (strong indigestion)	Digestive (45.30%) Found mainly in children, characterized by loss of appetite, indigestion, flatulency, tenesmus, abdominal pain and costiveness.
SUSTO (Fright) Espanto de tierra (soil's fright), susto de animales (animal's fright), susto de agua (water's fright), espanto negro (black fright), espanto rojo (red fright), mal de espanto (fright disease).	Unspecific (39.32%) Characteristic of many American cultures. Caused by natural or supernatural situations. Disturbance of the emotional condition with a variety of symptoms and influenced by local habits and traditions. Usually associated with weakness, apathy, loss of appetite, anemia, intestinal parasitism, and immune deficiency.
CAIDA DE LA MOLLERA (Fall of the bregma or fontanel) Mollera sofocada (suffocated fontanel), salida de mollera (fontanel's gone out), mollera caída (fallen fontanel), mal de mollera (fontanel disease).	Digestive (36.33%) Characteristic symptom of a severe dehydration in children less than a year old. Corresponding to the fontanel sinking usually accompanied with diarrhea, fever or vomit. In this case, most probably due to infectious gastroenteritis.
AIRE (Air) Aire de animales (animal's air), golpe de aire (air hit), azote de aire de muerto (dead's air whip), mal de aire (air disease), aire de agua (water air), aire de basura (garbage air).	Unspecific (29.32%) Assumed to be the action of some harmful external entity not always physic (air), which penetrates or sticks to some body part. Usually associated with skin, respiratory or muscle-skeletal problems.
DAÑO (Damage) Brujería (witchcraft), mal puesto (set evil), maldad (wickedness), hechizo (bewitchment), posesión (possession).	Unspecific (16.17%) Cultural belief in witchcraft able to produce discomfort, injury, illness or damage to a victim of natural or supernatural procedures.
TORCEDURAS (Sprain)	Muscle-skeletal (12.06%) Usually related to luxation, twist and other extremity traumatism produced during job accidents.
ANGINAS (Angina)	Respiratory (9.61%) Currently tonsillitis.
LATIDO (Beat)	Cardiovascular (8.51%) Alterations in the rhythm or intensity of the sanguineous flow mainly of the vascular system.
ALFERESIA (Infant's epilepsy)	Nervous (7.42%) Old Spanish name used to describe convulsion, usually in children and independently of the origin.

One of the important results form this work was the knowledge that although México has several different ecological zones and environmental conditions and not all plants grow everywhere, different cultures used whatever natural resources they had around. Examples of this ecological regionalism are the damiana (*Turnera diffusa*) and the zoapatle (*Montanoa tomentosa*). Both were used to induce childbirth, to relieve amenorrhea and dismenorrhea symptoms, and to treat other problems related to the reproductive biology of women. Damiana grows mainly in desert and semi-desert lands, and its use was reported in the northern and central states of Baja California Norte and Sur, Sonora, Chihuahua, Coahuila, Sinaloa, Durango, Zacatecas, Nayarit, Jalisco, and Hidalgo. Zoapatle grows better in the central and southern parts of México. It was reported in Hidalgo, San Luis Potosí, Veracruz, Guanajuato, Querétaro, Michoacán, Estado de México, Tlaxcala, Puebla, Guerrero, Morelos, and Oaxaca. None of these plants was reported in Yucatán, where ecological conditions are quite different from the rest of the country. So, the collected data suggested the existence of a basic pool of medicinal plants known by most of the traditional therapists across the country, and used for the same or similar illnesses (Table 12.3).[3]

Another study, but limited to just four groups of Mexican cultures (Maya, Nahua, Zapotec, and Mixe) located in three Mexican states (Yucatán, Veracruz, and Oaxaca) in the south and southeast parts of the country, gave similar results. Data from this study were collected almost ten years after the IMSS-COPLAMAR program, confirming the constant and preferential use for herbal remedies by these cultures.[4]

Sesqui- and Dierpenes of Mexican Plants

The first Mexican substance searched for, found, and isolated based on old traditions, was the sesquiterpene hernandulcin from *Lippia dulcis* Trev. It was determined by a human taste panel to be more than 1000 times sweeter than sucrose. The structure of this sesquiterpene was determined and confirmed by chemical synthesis. It was nontoxic when administered orally to mice, and did not induce bacterial mutation.[5] Further work has been carried out with some of the plants more frequently used (Table 12.3). The sesquiterpene alkaloids hippocrateine I, hippocrateine II, and emarginatine were identified in *Hippocratea excelsa* used in Mexican traditional medicine, and antimicrobial abietane type diterpenes were isolated from *Salvia albocaerulea*.[6,7]

Plants were also used by pre-Colombian cultures for religious ceremonies. This practice was closely allied to cures and health care. One example is the Mazateca group of Oaxaca, which used a vision-inducing mint, *Salvia divinorum*

Table 12.3: Basic Pool of Medicinal Herbal Resources in México. Program IMSS-COPLAMAR 1984

GROUP 1 **Herbal resources for gastrointestinal diseases**	
Antiparasites	
Chenopodium ambrosioides L.	(epazote)
Artemisia ludoviciana Nutt	(estafiate)
Cucurbita pepo	(calabaza-semillas)
Ficus galbrata	(leche de higeronia)
Castela nicholsoni	(chaparro amargoso)
Spasmolytics and digestives	
Matricaria recutita L.	(manzanilla)
Mentha piperita	(hierbabuena)
Chrysantemum parthenium L.	(Santa María)
Cinnamomum zeylanicum	(canela)
Tagetes lucida	(pericón)
Marrubium vulgare	(marrubio)
Foeniculum vulgare	(hinojo)
Laxative	
Ricinum communis	(ricino)
Tamarindus indica	(tamarindo)
Cassia fistula	(cañafístula)
Spondiam bombin	(ciruela)
Perezia adnata	(pipitzaoac)
Antidiarrhoeals	
Psidium guajava	(guayaba)
Persea americana	(aguacate)
Plantago major	(llantén)
Choleretics	
Taraxacum officinale	(diente de león)
Peumus boldus	(boldo)
GROUP 2 **Herbal resources for respiratory diseases**	
Antitussives	
Sambucus mexicana	(sauco)
Gnaphalium semiamplexicaule	(gordolobo)
Bouganvillea glabra	(bugambilia)
Allium sativum	(ajo)
Bronchodilators and antimicrobial	
Eucalyptus globulus	(eucalipto)
Thymus vulgaris	(tomillo)
Rosmarinus officinalis	(romero)
Mentha poleigum	(poleo)
Origanum vulgare	(orégano)
GROUP 3 **Herbal resources for skin and traumatisms problems**	
Antimicrobials	
Malva parviflora	(malva)
Verbena carolina	(verbena)
Euphorbia prostata	(hierba de la golondrina)
Anti-inflammatory	
Oenthera rosea	(hierba del golpe)
Hetherotheca inuloides	(árnica)
Cuphea aequipetala	(hierba del cáncer)
Cicatrizant	
Aloe barbadensis	(zábila)
Agave mexicana	(maguey)
Solanum hispidum	(sosa)
GROUP 4 **Herbal resources for cardiovascular diseases**	
Hypotensive	
Casimiroa edulis	(zapote blanco)
Sechium edule	(chayote)
Cardiotonics	
Talauma mexicana	(yoloxóchitl)
Chirantodendron pentadactylon	(flor de manita)
Tevethia tevetioides	(codo de fraile)
GROUP 5 **Herbal resources for diabetic and metabolic alteration's symptoms**	
Hypoglycaemics	
Opuntia sp	(nopal)
Tecoma stans	(tronadora)
Hypocholesterolaemia agents	
Guatteria gaumeri	(yumel)
Grataegus pubescens	(tejocote)

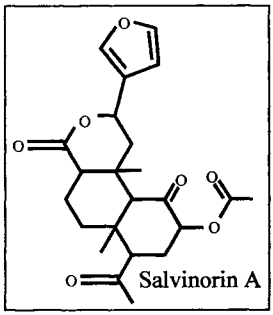
GROUP 6 **Herbal resources for urinary diseases**	
Diuretics	
Zea mays	(pelos de elote)
Equisetum hyemale	(cola de caballo)
Nephrolithiasis (calculi in the kidney)	
Larrea tridentata	(gobernadora)
Eryngium comosum	(hierba del sapo)

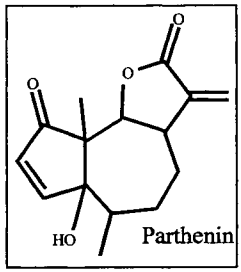
GROUP 7 **Herbal resources for malfunction of the feminine reproductive apparatus**	
Montanoa tomentosa	(zoapatle)
Ruta chalepensis	(ruda)
Lippia dulcis	(hierba dulce)
Petroselinum crispum	(perejil)
Turnera diffusa	(damiana)
Modified from Ref. 3	

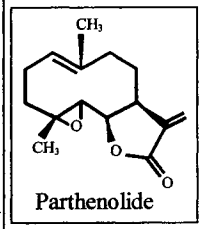
(Epling and Jativa-M) containing the neoclerodane diterpene divinorin A or salvinorin A (Fig. 12.1). It was the first documented non-alkaloidal diterpene hallucinogen. It is inactivated by the gastrointestinal system if orally ingested, and the effect is produced after absorption through the oral mucous.[8, 9]

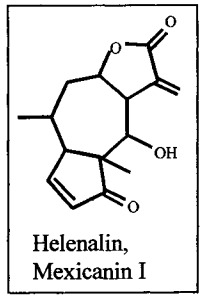
Among the most studied Mexican medicinal plants are the Asteraceae (Compositae). These plants produce a great number of useful compounds known mainly for their anti-inflammatory and muscular contraction effects. The natural products responsible are predominantly sesquiterpene lactones and diterpenes. These compounds are also cytotoxic and antitumoral, as shown by eupatoriopicrin and parthenin (Fig. 12.1). Special note is given to the *Parthenium* genus that produces many useful natural products such as rubber from guayule (*Parthenium argentatum*). Parthenolide (Fig. 12.1) from *Tanacetum parthenium* (feverfew) has anti-inflammatory activity permitting its use as an antimigraine agent and to relieve swelling in arthritis. However, this sesquiterpene lactone, constituent of European feverfew, may produce allergic contact dermatitis. Mexican samples of feverfew contain the eudesmanolid santamarin and traces of other sesquiterpene lactones, but parthenolide is not present.[10] Parthenin from *P. hysterophorus* is also an allergenic causing dermatitis, rhinitis, and hypotension. This plant is particularly rich in sesquiterpene lactones. Anti-ulcer activity has been found with ludartin, hymenin, 9-O-desacetylspathulin-2-O-angelate and mexicanin I or helenalin (Fig. 12.1). Helenalin from *Helenalin autumnale* increases cAMP by phosphodiesterase inhibition producing a Ca^{++} influx enhancing the contractility of the guinea-pig myocardium. Neurotoxicity is associated to repin and solstitialin cynaropicrin.[10]

Achillea millefolium has been used to relieve several discomforts such as cough, overeating, aches all over the body, and as a diuretic and emetic. Locally applied, it is effective for scabies and itching, a tonic for nervous disorders, hemorrhage, and stomach colic among others. *Artemisa ludovicana* ssp is still in use as a fore vermifuge and as a stimulant to menstrual complaints. Sesquiterpenes such as germacranolides, eudesmanolides, and guaianolides were found in this plant, and


Salvinorin A


Parthenin


Parthenolide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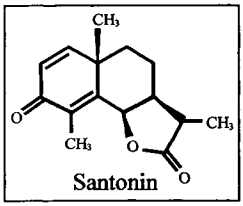
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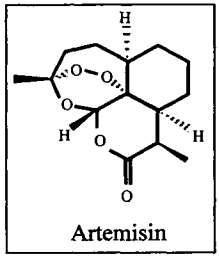
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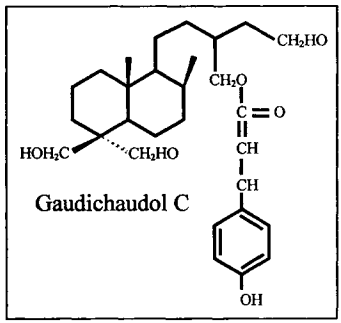
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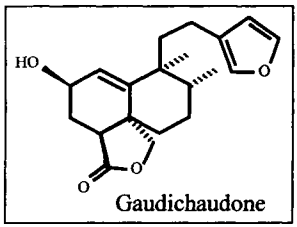
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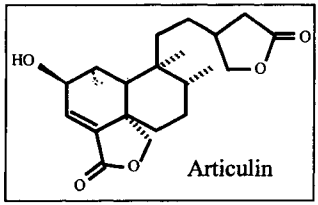
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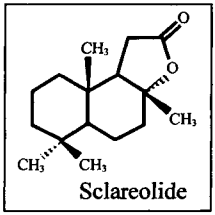
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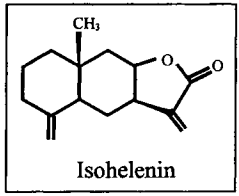
Isohelenin

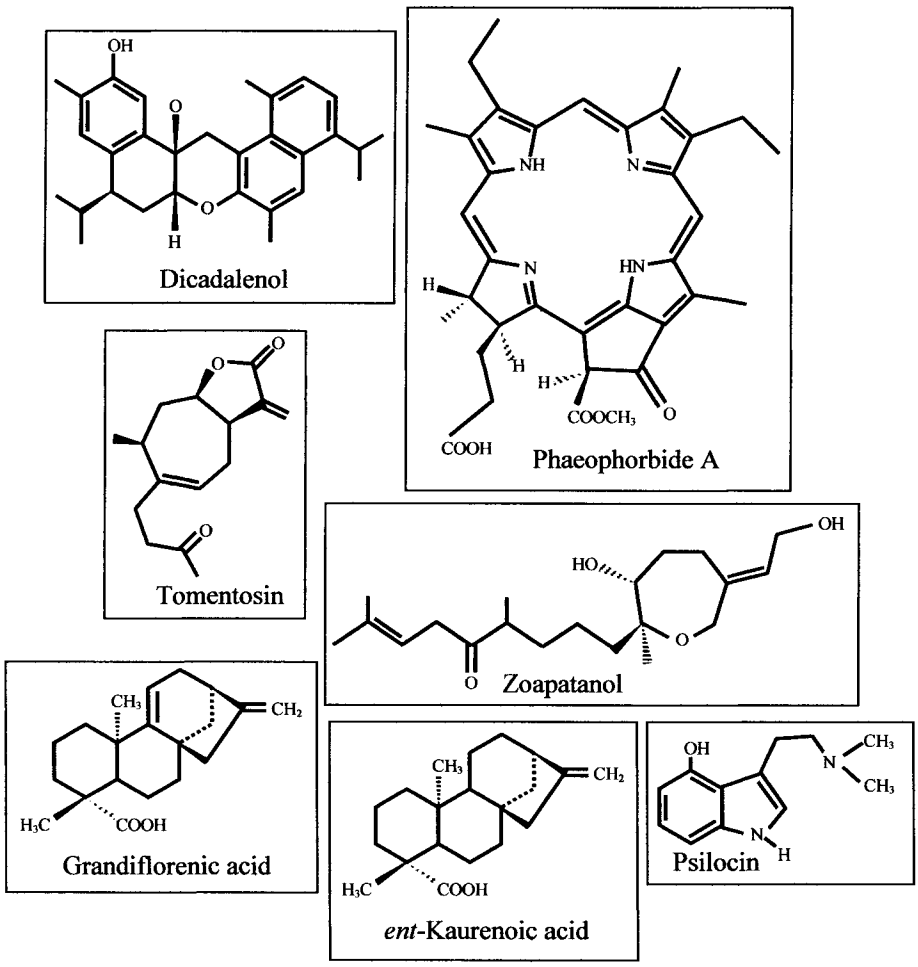

Figure 12.1: Chemical structures of some active terpenes.

the ethanolic extract strongly inhibits the anti-inflammatory transcription factor NF-κB.[11] The sesquiterpenes santonin and artemisin (Fig. 12.1) from *Artemisia tridentata* are used as anithelmintics. The *Baccharis* genus contains some cytotoxic diterpenoids, such as gaudichaudol C, gaudichaudone, and articulin (Fig. 12.1) from

B. gaudichaudiana, which is commonly used for diabetes. *Elephantopus scaber* contains hydroxylated germacranolides, such as molephantin and molephantinin, which cause diuresis, antipiresis, eliminate bladder stones, and have cytotoxic and antitumor properties. From the *Tithonia* genus, *T. diversifolia* is widely used in México and often has been identified erroneously as *Arnica montana.* Because of this confusion it is used as European arnica to treat hematomas and muscular cramps. The genus is rich in sesquiterpene lactones.[11]

ANTI-INFLAMMATORY MECHANISMS

In regard to terpenes, perhaps their most deeply studied, although not yet fully understood property, is their anti-inflammatory mechanism of action. Inflammation is the body's reaction to tissue injury or infection. The injured site becomes red and warm because of increased blood flow; swelling and fluffiness result from fluids seeping into local tissues, causing increased tissue tension. Chemicals involved in swelling also add to local pain. Within the inflamed area, special defense cells accumulate, including white blood cells, macrophages, and lymphocytes. The white blood cells break down the damaged tissue and signal macrophages; the latter ingest and digest foreign substances and dead tissue, in some diseases this process may be destructive to the host.

Most or all of these events are triggered by the activation of a nuclear factor called NF-κB. This is a pluripotent transcription factor. The target genes include cytokines, intercellular and vascular cell adhesion molecules, acute-phase proteins, and immuno-receptors. It also seems to be involved in apoptosis promotion, and is a key regulator of immune response genes.[12] Usually, NF-κB is a hetero-dimmer of p50 and p60 DNA-binding subunits. These two and another three different DNA-binding subunits share a N-terminal homology domain conferring DNA binding, dimerization, nuclear translocation, and interaction with inhibitory IκB proteins. Under normal conditions, NF-κB is retained in the cytoplasm by the two major isoforms of IκB proteins, IκB-α and IκB-β, which mask the NF-κB nuclear localization sequences. A variety of agents such as cytokines, UV irradiation, phorbol, and other pro-inflammatory stimuli induce the intracellular production of reactive oxygen species (ROS), which trigger a rapid degradation of IκB proteins by phosphorylation, ubiquitination, and the 26S proteasome pathway. This degradation activates the NF-κB by releasing the nuclear localization sequences. The transcription factor is then translocated into the nucleus starting the inflammatory gene expression.[12, 13]

Currently, it is known that the sesquiterpene lactones, parthenolide and isohelenin. (Fig. 12.1) do not interfere with ROS production and do not act directly on the DNA-binding subunits of NF-κB but rather prevent the induced degradation of IκB-α and IκB-β.[14] This stabilizes the respective binding with NF-κB, keeping the

transcription factor in the cytoplasm and avoiding translocation to the nucleus. However, the IκB subunits do not seem to be the direct target of sesquiterpene lactones, and it is still unclear which member or members of the signaling cascade are the real targets. Not all sesquiterpene lactones are inhibitors of NF-κB activation. Key structural elements of inhibitory sesquiterpene lactones are the isoprenoid ring and the lactone ring containing a conjugated exomethylene group in the α-position. These two elements form a reactive system allowing the nucleophilic addition of carbanions to α,β-unsaturated carbonyl compounds. This is known as the Michael addition. It is the target for nucleophilic substrates, as in parthenolide and isohelenin. Isoprenoids without the lactone ring or the exomethylene group do not have the same effect, as has been proven with santonin and sclareolide (Fig. 12.1). Parthenolide and isohelenin sesquiterpenes display a high degree of specificity for the inhibitory activity of NF-κB, showing no effect with other transcription factors. Neither interferes in an unspecific manner with other signaling molecules.[12]

Inflammation problems of many organs may be alleviated with sesquiterpenes, as shown by inhibition of chemokine interleukin 8 (IL-8) gene expression by parthenolide and isohelenin. The respiratory epithelium seems to be an important source of IL-8 in the lungs, and the respective expression is induced by the pulmonary macrophage-derived tumor necrosis factor α (TNF-α). Both factors (TNF-α and IL-8) are part of the cytokine network involved during acute lung injury, and are thought of as agents that amplify and prolong swelling.[13] The same inhibition of NF-κB activation, through the prevention of induced IκB-α and IκB-β degradation, resulted in the absence of nitric oxide (NO) in stimulated cultured rat aortic smooth muscle cells. NO is considered a regulatory and effector molecule in diseases involving inflammatory processes. It is produced by nitric oxide synthase (NOS), which is usually present in several isoforms in mammals. Neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) are some of the isoforms, and iNOS is expressed in response to proinflammatory signals. iNOS is responsible for prolonged and high output of NO. Neither NO production nor iNOS-mRNA accumulation were detected after sesquiterpenes treatment in a concentration-dependent manner. Additionally, transient transfection experiments using an iNOS promoter-luciferase reporter plasmid demonstrated that parthenolide and isohelenin inhibited activation of the iNOS promoter.[15]

Recently, two new sesquiterpene lactones of the furanoheliangolide type were reported from *Neurolaena macrocephala*. This plant is found in tropical areas of Veracruz, México but no mention of anti-inflammatory properties were noted. Despite the different sesquiterpene rings in comparison to parthenolide, some NF-κB inhibition is expected, because all furanoheliangolides reported thus far from this plant contain the lactone ring and the exomethylene group in the α-position.[16] Three new compounds were also isolated from *Heterotheca inuloides* (Mexicana arnica). One of these, dicadalenol (Fig. 12.1), with a molecular formula $C_{30}H_{36}O_3$ and a

molecular ion at 444.6121, showed significant dose-dependent anti-inflammatory activity against TPA-induced mouse ear edema. The NMR spectrum and stereochemical studies did not show a lactone ring or exomethylene group, but a particular pseudo-axial orientation of the C(4)-C(11) σ-bond was proposed as forming a pseudo-boat form for the cyclohexene ring. A β-orientation of the C(4)-C(11) bond is usually seen in sesquiterpenoids from Asteraceae.[17] Whether or not this conformation results in a reactive system suitable for Michael addition of nucleophilic substrates and potentially NF-κB inhibitor, is as yet unknown. Another sesquiterpenoid was previously isolated from this plant, 7-hydroxy-3, 4-dihydrocadalin, and was evaluated as an antioxidant because it inhibited the mitochondrial and microsomal lipid peroxidation.[18] Cytotoxic and anti-inflammatory labdane and *cis*-clerodane diterpenes were also identified in other plants. Labd-13(*E*)-ene,-8 alpha,15-diol, and labd-13(*E*)-ene,-8 alpha,15-yl acetate were isolated from *Cistus creticus* subsp. *eriocephalus* (Viv.), while (+)-19-acetoxy-*cis*-clerodan-3-en-15-oic acid was isolated from *Cistus monspeliensis* L.[19]

In the case of diterpenes, a different mechanism may be involved in anti-inflammatory activity. Interference with DNA-binding activity of p50 protein of NF-κB factor seems to be a mechanism, as shown with kamebakaurin, a kaurane diterpene. This compound did not prevent degradation of IκB-α isoform or nuclear translocation of NF-κB factor, and the covalent modification of cysteine 62 of p50 by kamebakaurin was proven. Prevention of the induced expression of anti-apoptotic genes and an increase in the induced caspase 8 activity were also observed. These properties make this kaurane diterpene a good candidate for inflammation and cancer treatment.[20]

Researchers continue looking for other useful effects of terpenoids such as the interactions of six diterpenes (three clerodanes, two abietanes, and one rosane) with the cyclooxygenase and 5-lipoxygenase pathways of arachidonate metabolism and nitric oxide production.[21] The elucidation of mechanisms may help to design new drugs for several diseases. Efforts toward this are already fruitful, as evidenced by the chemical synthesis of the potent anti-inflammatory agent (-)-acanthoic acid. This was achieved by an enantioselective synthesis starting from (-)-Wieland-Miescher ketone. This stereocontrolled procedure opens attractive alternatives to synthesize biologically active diterpenes.[22] Finally, phaeophorbide A (Fig. 12.1), a degradation product from chlorophyll and quite different from sesquiterpene lactones and diterpenes, was shown to interfere with the NF-κB activation. Because it was cytotoxic under light but harmless in the dark, the results are still controversial. It was isolated from *Solanum diflorum* Vell used in Oaxaca, México to treat erysipelas, local swellings, edema, and fever.[23]

CIHUAPAHTLI, WOMAN'S MEDICINE

Pharmacological Effects

The "cihuaphatli" plant is one of the most used by old Mexican cultures, mainly from the central and southern areas of the country where it grows. "Cihuaphatli" is the medicine for women because of its uterotonic properties. It has long been used to induce childbirth and to regulate the menstruation period and it is still commonly used among country people. It has been proposed as a natural contraceptive. Chemical studies on zoapatle started in the nineteenth century, but the isolation of pure substances was not accomplished until 1970 when root derivatives of kaurenic acid were found.[24] Tomentosin (Fig. 12.1), the first sesquiterpene lactone isolated from zoapatle, was reported in 1971. In 1972, the search of other substances was initiated and the isolation of montanol and zoapatanol (Fig. 12.1) was achieved.[25,26] These studies attracted commercial interest. Six patents (3,986,952, 3,996,132, 4,046,882, 4,060,604, 4,112,078, and 4,237,053) for the extraction procedures, chemicals rights, and synthesis of these substances were processed in the U.S.A and acquired by the Ortho Corporation belonging to the Johnson & Johnson Company between 1976 to 1980. Unfortunately, zoapatanol and montanol were not the active substances. Another patent (4,006,227) for a pharmaceutical composition, useful for controlling fertility, and obtained by extraction from *Montanoa tomentosa*, was requested by the Mexican team studying the biological activity of zoapatle extracts. This request was unresolved and abandoned after 5 years.

In 1974, 17-hydroxy-*ent*-kaur-15-en-19-oic acid and grandiflorenic acid (Fig. 12.1) compounds similar to those found in zoapatle were reported from *Aralia cordata* Thunb.[27] During the 80's, intense research on zoapatle active substances was initiated, and clinical trials on women treated with zoapatle extracts were carried out.[28-32] Kauradienoic acid was isolated, and the uterotonic property was reported in different animal tissues *in vivo* and *in vitro* and compared to the verapamil effect.[33,34] A weak effect of kaurenoic acid (Fig. 12.1) isolated from *Montanoa frutescens* and another two derivatives on the motility and viability of human spermatozoa was reported.[35] Interestingly, an ambivalent myometrial response to zoapatle leaves aqueous extract (ZACE) was observed, depending on the animal species and on the existence or absence of pregnancy. The aqueous crude extract of *Montanoa tomentosa* leaves produced different (or even opposite) effects on uterine motility during the distinct phases of the estrus cycle in the rat. Furthermore, pure grandiflorenic acid (GA) reproduced the uterine responses induced by ZACE.[36,37]

In addition to these observations, the 16-α-hydroxy-*ent*-kauran-19-oic acid, isolated from *Montanoa hibiscifolia*, and its methyl ester inhibited spontaneous oxytocin- and potassium-induced contractile activities. The methyl ester inhibition was much stronger, 2 – 15 times more than the acid at μM concentrations. This

inhibitory effect was confirmed by adding kaurenoic acid (*M. frutescens)*, grandiflorenic, and kauradienoic acid (*M. tomentosa*), and 16-hydroxy-*ent*-kauran-19-oic acid and its methyl ester (*M. hibiscifolia*) to rat uterus whose contractile activity was induced by acetylcholine, oxytocin, and serotonin. All substances inhibited contractions through a mechanism independent of both 2-adrenergic and H2-histaminergic receptors present in uterine smooth muscle.[38, 39] The possibility of different effects in response to these substances may be related to the physiological or estrogenic condition of the animal tissues at the time of the assay and/or to the metabolic condition of the zoapatle plants when collected. It is widely believed that many plants respond and adapt to changes in the surrounding ecosystem with changes in secondary metabolites. It is also known that zoapatle plants from different varieties, lose or decrease their active substances concentrations when transplanted from their normal environment to a new place.[40] However, it is also possible that substances having opposite effects may be present under normal conditions.

There are other plants containing similar terpenes but that have relaxing effects on the same or different muscle tissues. Xanthorrhizol, xanthorrhizol glycoside, and trachylobanoic acid, isolated from medicinal plants known as Cachani complex, inhibit the tonic contraction of rat uterus induced by depolarizing potassium solution, $CaCl_2$, and BAY K 8644 in a concentration-dependent manner.[41] Also, *ent*-kaurenoic acid isolated from *Viguiera robusta* (Asteraceae) was tested for activity on vascular smooth muscle contractility, and, at high concentrations (20 g/mL), inhibited the *in vitro* contractility of rat carotid artery elicited by phenylephrine, but had no effect at lower concentrations.[42] Apparently, these compounds induce contraction in naturally relaxed muscles, but if the tissue is under spasmodic stimuli, then the effect is the opposite. Additional research has been carred out to solve potential detection problems that might be related to these apparently opposite effects.[43] It is likely that the uterotonic effect helps some organisms, as female chimpanzees consume *Aspilia* species more frequently than male animals. The diterpenes kaurenoic and grandiflorenic acids have been isolated from *Aspilia mossambicensis* leaves.[44]

Encelin from *Montanoa speciosa* is inhibitory to growth and development of fungal cells of *Mucor rouxii*. Other cytotoxic sesquiterpene lactones from *Montanoa tomentosa* ssp. *microcephala* have been reported.[45,46] A number of kaurane diterpenes and *ent*-kauranoids have been reported that are potentially useful because of their cytotoxicity against human tumor cells, anti-HIV activity, and trypanosomicidal activity.[47-56] New structures are frequently discovered, and probably we will learn new effects in humans and animals in the future.[57,58] Paradoxically, these plant natural products probably have specific and important effects in the plants, but we are still ignorant of these roles.

Biochemistry and Biotechnology of Active Substances

Biosynthesis (Fig. 12.2) of kaurene diterpenes starts from the condensation of D-glyceraldehyde-3-phosphate and pyruvate to produce 1-deoxy-D-xylulose 5-phosphate (DXP), which is then transformed into isopentenyl pyrophosphate (IPP), the 5 carbon (5C) building block of all terpenes. IPP also is synthesized from acetyl-CoA via mevalonic acid (MVA) or mevalonate, the most well-known terpene biosynthetic pathway. Generally, the MVA pathway produces cytoplasmic sesquiterpenes, *e.g.*, capsidiol of the Solanaceae, and triterpenes as sterols. The DXP pathway produces the plastidic monoterpenes, such as essential oils, diterpenes like gibberellins and kaurenes, and tetraterpenes such as carotenoids. Some exchange of precursors and intermediates seems to exist between both pathways. Once IPP is formed, the next step is condensation with its isomer dimethylallyl pyrophosphate (DMPP) to produce geranyl pyrophosphate (GPP), a 10C unit and immediate precursor of monoterpenes. A further condensation reaction of IPP to the growing carbon chain produces a 15C unit or farnesyl pyrophosphate (FPP), the immediate precursor of sesquiterpenes by cyclization, and triterpenes (30C) by joining of two FPP molecules. Addition of IPP to FPP yields a 20C unit of geranylgeranyl pyrophosphate (GGPP), the immediate precursor of diterpenes by cyclization, and tetraterpenes (40C) by joining of two GGPP molecules.[59] Further IPP condensation continues to produce longer terpenes, such as rubber. From *trans*-GGPP, a ring closure is carried out by the *ent*-copalyl diphosphate synthase (CPS) to produce *ent*-copalyl diphosphate. Then, the PP moiety is released, and the kaurene ring is formed by the *ent*-kaurene synthase (KS) yielding *ent*-kaurene. These are proposed as plastidic reactions, and once the *ent*-kaurene is released to the plastid, it is oxidized by *ent*-kaurene-19-oxidase (EKO) to produce the *ent*-kaurenoic acid (KA).[60,61] Further hydroxylation, reduction, and oxidation reactions of KA result in gibberellin biosynthesis. Specific desaturation reactions of KA may be involved in the double bond formation characteristic of the active compounds of zoapatle, such as monogynoic (MO) and grandiflorenic (GA) acids. In our lab, we established that zoapatle contains high concentrations of kaurene diterpenes in all tissues during all developmental stages both *in planta* and *in vitro*. These are accumulated mainly in glandular trichomes on the leaf surfaces. This is probably why these substances are hardly detected in cell suspension and callus tissue cultures. Leaf wounds seem to transiently decrease the kaurene diterpenes production. Genetic transformation of zoapatle cells is potentially feasible but still hard to achieve.

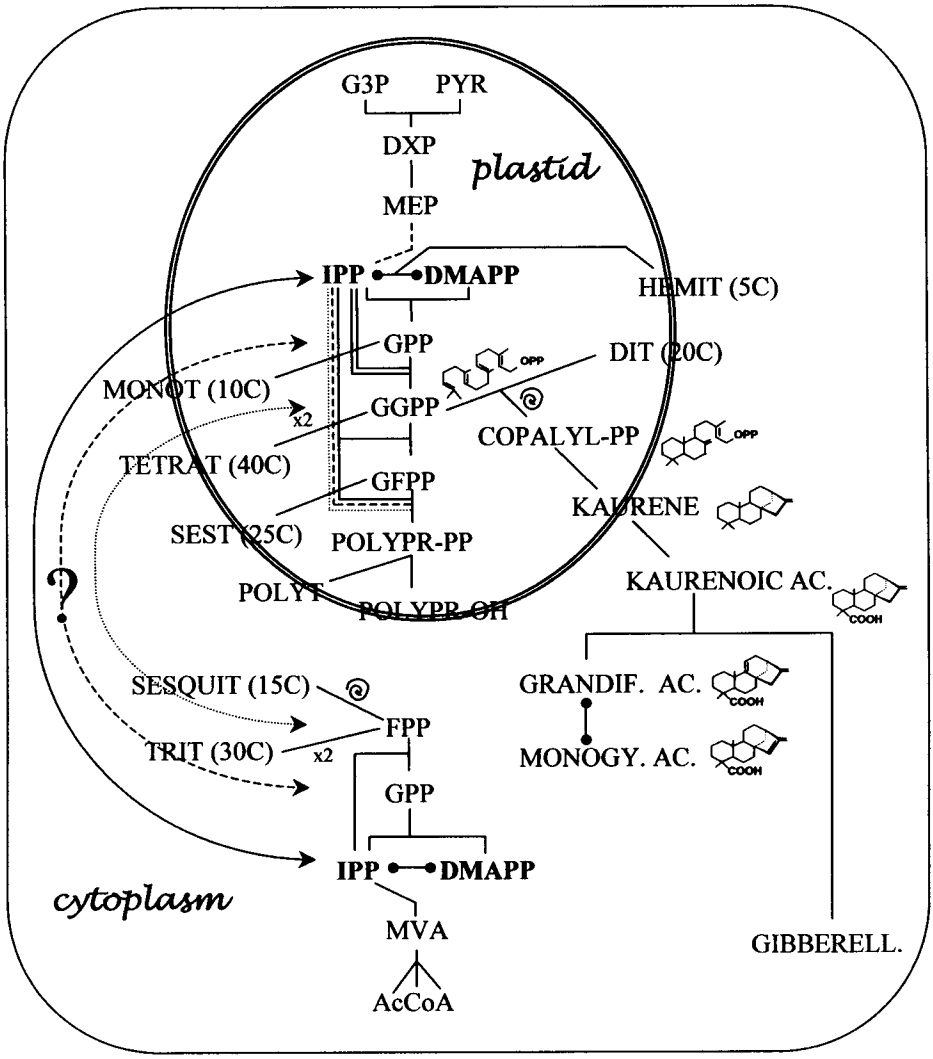
G3P
PYR
DXP
plastid
MEP
IPP
DMAPP
HEMIT (5C)
GPP
DIT (20C)
MONOT (10C)
GGPP
x2
COPALYL-PP
TETRAT (40C)
GFPP
KAURENE
SEST (25C)
POLYPR-PP
KAURENOIC AC.
POLYT
POLYPR-OH
?
SESQUIT (15C)
GRANDIF. AC.
FPP
TRIT (30C)
x2
MONOGY. AC.
GPP
IPP
DMAPP
cytoplasm
MVA
GIBBERELL.
AcCoA

Figure 12.2: Plastidic and cytoplasmic pathways of active zoapatle diterpenes.
(AcCoA) acetyl-CoA, (COPALYL-PP) copalyl pyrophoaphate, (DIT) diterpenes, (DMAPP) dimethylallyl pyrophosphate, (DXP) 1-deoxy-D-xylulose-5-phosphate, (FPP) farnesyl pyrophosphate, (G3P) D-glyceraldehyde-3-phosphate, (GGPP) geranylgeranyl pyrophosphate, (GIBBERELL.) gibberellins, (GPP) geranyl pyrophosphate, (GRANDIF. AC) grandiflorenic acid, (HEMIT) hemiterpenes, (IPP) isopentenyl pyrophosphate, (MEP) 2-C-methyl-D-erythritol-4-phosphate, (MONOGY. AC) monogynoic acid, (MONOT) monoterpenes, (MVA) mevalonic acid, (POLYPR-OH) polyprenols, (POLYPR-PP) polyprenyl pyrophosphates, (POLYT) polyterpenes, (PYR) pyruvate, (SESQUIT) sesquiterpenes, (SEST) sesterpenes, (TETRAT) tetraterpenes, (TRIT) triterpenes, (x2) indicates joining of two molecules and ( ) indicates a cyclization reaction. Arrows suggest putative exchange of precursors and intermediates between plastids and cytoplasm. Substances crossing the limit of the plastid indicate possible excretion or release into the cytoplasm. Bold characters indicate the building blocks of all terpenes.

CURRENT MEDICINAL PLANT USE

Cultural Differences

When medicinal plants are used by foreign cultures different from those where the plants originated, the result may be the discovery of other plant properties or the misuse of the plant attributes. Many Mesoamerican plants are currently used, and some are indispensable, in Occidental kitchen and food recipes. These include tomato, vanilla, cacao seeds as chocolate, peppers, potatoes, and many others. However, development of medicinal plant traditions and legacy is not at a scientific level. Biotechnology is discovering new medicines and remedies, and drug design is improving based on ancient knowledge, but business is applying this knowledge sometimes with important consequences. Among the best examples are the saponins found in huge amounts in wild yam also called "cabeza de negro" (*Dioscorea composita*), a plant widely distributed in the past in México. Saponins of this plant supply the basic nucleus for several steroidal hormones production. This plant has been used by many industries for massive oral contraceptive production because total synthesis is not cost-effective. Therefore, production required reliable supplies of plant material, either from plantages or from the wild. This is the case of the wild yam, which cannot be cultivated and is exported from México and other countries in quantities of hundreds of tons. Because of the drastic demand for wild yam, and the

availability of the final products, a significant change in human behavior and cultural development has occurred. [62-70]

Larrea tridentata, a desert plant found in northern México and southern U.S.A., also called "gobernadora", "hediondilla", "creosote", or "chaparral", has been used for a long time because of its many different properties, such as an antioxidant, an aging retardant, for weight loss, as a liver tonic, as an anticancer agent, and as a blood purifier. Health problems associated with this plant are known in the U.S.A., such as severe hepatitis requiring orthotopic liver transplantation. The apparent reason is non-traditional use. Affected people were taking *Larrea* extracts in the form of tablets, as capsules, in teabags, and in many cases together with other medicaments like lovastatin, acetaminophen, and even alcohol. Also, these extracts were taken for long periods of time as "natural preventives". [71-73] The availability of herbal products as "over-the-counter" drugs and their increasing use have caused concern to the respective authorities, since these products are not currently monitored for their safety, efficacy, and quality. Reliable information on these aspects is not available. Most of the clinical trials carried out to date have been lacking in scientific design, as well as in data collection, interpretation, and systematic toxicological evaluation. A critical review of the existing data from many plants such as ginseng, "uña de gato," and others is necessary.[74-76]

Confusion of plant names with similar plants is also producing health problems as a consequence of "borrowed" medicinal traditions. Health problems have been reported by ingestion of "gordolobo" tea. This is an imported plant from México normally used for cough and pulmonary problems. Unfortunately, the tea used in the U.S. was prepared with *Senecio longilobus* or *S. douglasii*, both rich in pyrrolizidine alkaloids. The Mexican gordolobo (*Gnaphalium macouni*) is a similar plant but without the toxic substances. Both plants are desert bushes, have woody stems and gray hairy leaves, but only professionals and healers know and use the right one.[77-80] A similar confusion was reported with alpendost (*Adenostyles alliariae*), which contains the pyrrolizidine alkaloids seneciphylline and the corresponding N-oxide, instead of the non-harmful coltsfoot (*Tussilago farfara*). The two plants can easily be confused especially after the flowering period.[81] Several hepatic problems have been reported.[82-85]

Another consequence of the use of ethnobotanical material by a culture different from the original one, is the misuse of this material. The hallucinogenic mint *Salvia divinorum* has been used in Oaxaca, México for many years for curing and divination. It contains the non-alkaloidal *trans*-neoclerodane diterpenes salvinorin or divinorin A, B, and C and is used as substitute of smoked marijuana.[8,9,86-88] Specimens of *S. divinorum* have been seized in large-scale illicit in-door and out-door hemp plantations, and more plants have been discovered in horticultural greenhouses. Because neither the magic mint nor its active compounds are banned substances, their use as recreational legal drugs may be increasing.[89] The discovery of the hallucinogenic lysergic acid or LSD (from German, Lysergsäure

Diethylamid) in the Mexican morning glory species or the Aztec "ololouqui," and psilocin (Fig. 12.1) in the sacred but hallucinogenic mushroom "teonanacatl" (*Psilocybe aztecorum*) used by Maria Sabina, the famous Mexican chaman, are now exploited in recreational use. Other examples of recreational drugs are also documented.[90-92]

Potential problems and consequences of "borrowed" traditions from medicinal plants are described by Huxtable as follows: Right plants are used in wrong ways as concentrates, capsules, and tablets resulting in a higher dose and a concentrated mixture of active substances. The same plants are used differently (as preventative rather than as treatment), resulting in a regular rather than an intermittent use and leading to poisoning by accumulative and toxic concentrations. Plant material is used outside of the original cultural context without the advice of ethnic practitioners, resulting in a wrong and dangerous practice. Right plants may be used in combination with other pharmacologically active agents such as alcohol, caffeine, or recreational drugs, resulting in adverse herb-drug interactions.[73] Because people easily and rapidly move around the world, the use of herb based "natural" cures is growing worldwide. Thus, it is even more important to consider that the potential presence of patients from many different cultural backgrounds may result in unknown practices of serious consequences.

SUMMARY

This chapter summarizes the current knowledge of a small part of the Aztec knowledge concerning some sesqui- and diterpenes from Mexican medicinal plants. New anti-inflammatory drugs are being designed based on the mechanism of action of some of these, and other medicines are still to be developed once are able to elucidate their mechanisms. Finally, the current cultural differences produced by the use and abuse of these plants outside of their original cultural context is discussed.

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